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☐ 1. Document ID: US 20040241856 A1

L4: Entry 1 of 10 File: PGPB Dec 2, 2004

DOCUMENT-IDENTIFIER: US 20040241856 A1
TITLE: Methods and compositions for modulating stem cells

Summary of Invention Paragraph:
[0006] In one aspect, the invention provides methods for inhibiting differentiation of mammalian stem cells. The methods entail (a) providing a population of stem cells, (b) introducing a vector comprising an HSC differentiation-inhibiting polynucleotide of the present invention into the stem cells, and (c) expressing a polypeptide encoded by the polynucleotide by culturing the modified stem cells, thereby inhibiting differentiation of the stem cells. In some of the methods, the stem cells are isolated from bone marrow. In some preferred methods, the stem cells are human hematopoietic stem cells. The human stem cells can be first selected for expression of CD38 and Thy prior to introduction of the vector. In some of the methods, the HSC differentiation-inhibiting polynucleotide encodes GATA-binding protein 3 or ID3.

Summary of Invention Paragraph:
[0007] In a related aspect, the invention provides methods for increasing the effective dose of hematopoietic stem cells in a mammalian subject. The methods require (a) providing a population of hematopoietic stem cells, (b) introducing into the cells an HSC differentiation-inhibiting polynucleotide of the present invention, and c) administering the genetically modified cells that express an HSC differentiation-inhibiting polypeptide to a mammalian subject; thereby increasing the effective dose of hematopoietic stem cells in the subject. In some of these methods, the administered stem cells are a subpopulation of the modified cells that are selected for expression of the polypeptide prior to administering to the subject. In some preferred methods, the subject is human, and the hematopoietic stem cells are human hematopoietic stem cells. In these methods, the hematopoietic stem cells can be selected for expression of CD34 and Thy prior to introducing into the cells the HSC differentiation-inhibiting polynucleotide.

Summary of Invention Paragraph:
[0008] In another related aspect, the present invention provides methods for inhibiting hematopoietic stem cell differentiation using an HSC differentiation-inhibiting polypeptide identified by the present inventor. The methods entail contacting a population of HSCs with an effective amount of the HSC differentiation-inhibiting polypeptide which inhibits differentiation of the HSCs. In some of the methods, the HSCs are present in an in vitro cell culture. In some other methods, the HSCs are present in a subject grafted with the HSCs. In some preferred methods, the subject is human.

Summary of Invention Paragraph:
[0009] In another aspect, the invention provides methods for isolating a population

of cells that are enriched for hematopoietic stem cells (HSCs). These methods comprise (a) obtaining a sample of cells containing hematopoietic stem cells, (b) selecting cells from the sample based on expression or lack of expression of at least one known HSC surface marker, and at least one novel HSC molecule marker identified in the present invention, and (c) separating cells with the known HSC marker and at least one of the novel molecule markers; thereby isolating a population of human cells enriched for hematopoietic stem cells.

Summary of Invention Paragraph:
[0010] Preferably, the hematopoietic stem cells enriched with these methods are human HSCs. In some methods, the known human HSC marker is CD34+ and Thy+. In some of the methods, the at least one novel HSC marker is a human HSC surface molecule identified in the present invention.

Summary of Invention Paragraph:
[0011] In another aspect, the invention provides methods for enumerating hematopoietic stem cells in a population of cells. The methods entail (a) contacting the population of cells with an antibody that specifically binds to one novel HSC surface marker identified in the present invention under conditions that allow the antibody to specifically bind to the HSC surface marker, and (b) quantifying the cells recognized by the antibody; thereby enumerating hematopoietic stem cells in the population of cells. In some of these methods, the hematopoietic stem cells are human HSCs, and the population of cells are first selected for expression of CD34 and Thy prior to the contacting.

Detail Description Paragraph:
[0017] Using HSCs enriched from blood of normal human donors, it was found that sequences upregulated in the human HSCs include genes encoding hormones, enzymes, histone, transcription factors, secreted proteins, surface markers, and other molecules. Table 1 lists examples of these genes that are upregulated in human HSCs (CD4+Thy+) as compared to non stem cells (CD4+Thy-). Further, using HSCs isolated from two different sources, bone marrow and peripheral blood, the present inventor identified a set of genes that are differentially expressed in HSCs from both sources. Some of these genes are shown in Table 2.

Detail Description Paragraph:
[0021] As indicated by the GenBank accession numbers or other identification numbers or descriptions in Tables 1, 3, and 4, sequences of the upregulated human and mouse HSC genes disclosed herein are all known in the art. Thus, as detailed below, the HSC differentiation-inhibiting polynucleotide sequences can be easily obtained commercially, from the sources disclosed in the public databases, or isolated using routine techniques of molecular biology. The encoded polypeptides can also be obtained commercially or easily produced with standard procedures of recombinant techniques.

Detail Description Paragraph:
[0022] The invention also provides methods for isolating and enriching HSCs. The currently known HSC markers are not satisfactory because they cannot accurately predict homogeneity and hematopoiesis activities of cells bearing the markers. The discovery of genes differentially expressed in HSCs provides novel molecular markers for selecting and enriching HSCs. For example, antibodies against novel surface markers disclosed in the present invention (e.g., those in Tables 2, 3, 4 and 5) can be used to isolate human and mouse HSCs from a crude population of cells (e.g., bone marrow or peripheral blood). The methods can also be directed to cell populations already enriched for one or more of the known HSCs makers (e.g., CD34+, Thy+ in human, and CD38+, c-kit+, Sca1+ in mice). Further enrichment using these novel markers can lead to more homogeneous HSCs with more potent hematopoiesis activities.

Detail Description Paragraph:

[0031] An "effective amount or dose" is an amount sufficient to effect beneficial or desired results. An effective amount may be administrated in one or more administrations. Determination of an effective amount is within the capability of those skilled in the art. Particularly preferred subjects of the invention in general include living mammals such as human, mice and rabbit, most preferred are humans. The administration of an HSC differentiation-inhibiting polypeptide, or a genetically modified cell comprising a polynucleotide sequence of the invention, may be by conventional means, for example, injection, oral administration, inhalation and others. Appropriate carries and diluents may be included in the administration of the polypeptide or the modified cells. Samples including the modified cells and progeny thereof may be taken and tested to determine transduction efficiency.

Detail Description Paragraph:
[0045] In addition to novel markers and methods for isolating HSCs, the invention also provides methods for inhibiting or blocking differentiation of mammalian hematopoietic stem cells, thereby promoting expansion of the stem cells. A number of the novel HSC marker genes identified in the present invention can inhibit or block HSC differentiation. Examples of such differentiation-inhibiting genes are shown in Tables 1 and 2 (for human HSC) and Tables 3 and 4 (for mouse HSC). For example, as described in the Examples below, human stem cells overexpressing GATA-binding protein 3 slows differentiation of the cells. HSCs overexpressing ID3 increased colony forming cells, indicating enhanced HSC activity as compared to a control. These differentiation-inhibiting molecules can be used in the present invention to inhibit HSC differentiation and thereby promoting expansion in vitro. They can also be used in vivo to increase the effective dose of engrafted HSCs in a subject.

Detail Description Paragraph:
[0046] The term HSC differentiation-inhibiting molecules (polynucleotides and the encoded polypeptides) include the molecules shown in Tables 1-4 that inhibit or slow HSC differentiation. Polynucleotides with substantial sequence identity are also encompassed. In addition, they also include variants, analogs, fragments, or functional derivatives of the HSC differentiation-inhibiting molecules shown in Tables 1-4. These differentiation-inhibiting molecules can be obtained from any species. Preferably, they are from mammalian species including human, mouse, and chicken. The HSC differentiation-inhibiting molecules can also be from any source whether natural, synthetic or recombinant.

Detail Description Paragraph:
[0050] Other than using a differentiation-inhibiting polypeptide, inhibition of HSC differentiation can also be achieved using an HSC differentiation-inhibiting polynucleotide to genetically modify HSCs. HSC differentiation-inhibiting polynucleotides suitable for these methods include some of the genes upregulated in HSCs (as shown in Tables 1 and 3). They encode HSC differentiation-inhibiting polypeptides that block or slow down differentiation of the HSC cells. Some of these methods require first isolation of a population of hematopoietic cells, e.g., a population of CD34.sup.+Thy.sup.+ human cells or CD34.sup.-CD38.sup.+ mouse cells as described above, from a source of such cells. An HSC differentiation-inhibiting polynucleotide of the invention can then be introduced into the cells whereby the cells are genetically modified.

Detail Description Paragraph:
[0052] Genetic modification as used herein encompasses any genetic modification method of introduction of an exogenous or foreign gene into mammalian cells (particularly human stem cell and hematopoietic cells). The term includes but is not limited to transduction (viral mediated transfer of host DNA from a host or donor to a recipient, either in vitro or in vivo), transfection (transformation of cells with isolated viral DNA genomes), liposome mediated transfer, electroporation, calcium phosphate transfection or coprecipitation and others.

Methods of transduction include direct co-culture of cells with producer cells (Bregni et al., Blood 80:1418-1422, 1992) or culturing with viral supernatant alone with or without appropriate growth factors and polycations (Xu et al., Exp. Hemat. 22:223-230, 1994).

Detail Description Paragraph:
[0056] Preferred vectors include retroviral vectors (see, Coffin et al., "Retroviruses", Chapter 9 pp; 437-473, Cold Springs Harbor Laboratory Press, 1997). Vectors useful in the invention can be produced recombinantly by procedures well known in the art. For example, WO94/29438, WO97/21824 and WO97/21825 describe the construction of retroviral packaging plasmids and packing cell lines. Exemplary vectors include the pCMV mammalian expression vectors, such as pCMV6b and pCMV6c (Chiron Corp.), pSFFV-Neo, and pBluescript-Sk+. Non-limiting examples of useful retroviral vectors are those derived from murine, avian or primate retroviruses. Common retroviral vectors include those based on the Moloney murine leukemia virus (MoMLV-vector). Other MoMLV derived vectors include, Lmily, LINGFER, MINGFR and MINT (Chang et al., Blood 92:1-11, 1998). Additional vectors include those based on Gibbon ape leukemia virus (GALV) and Moloney murine sacroma virus (MOMSV) and spleen focus forming virus (SFFV). Vectors derived from the murine stem cell virus (MESV) include MESV-MiLy (Agarwal et al., J. of Virology, 72:3720-3728, 1998). Retroviral vectors also include vectors based on lentiviruses, and non-limiting examples include vectors based on human immunodeficiency virus (HIV-1 and HIV-2).

Detail Description Paragraph:
[0057] In producing retroviral vector constructs, the viral gag, pol and env sequences can be removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by foreign DNA are usually expressed under the control a strong viral promoter in the long terminal repeat (LTR). Selection of appropriate control regulatory sequences is dependent on the host cell used and selection is within the skill of one in the art. Numerous promoters are known in addition to the promoter of the LTR. Non-limiting examples include the phage lambda PL promoter, the human cytomegalovirus (CMV) immediate early promoter; the U3 region promoter of the Moloney Murine Sarcoma Virus (MMSV), Rous Sacroma Virus (RSV), or Spleen Focus Forming Virus (SFFV); Granzyme A promoter; Granzyme B promoter, CD34 promoter; and the CD8 promoter. Additionally inducible or multiple control elements may be used.

Detail Description Paragraph:
[0063] Typically, the host cells for expressing the HSC differentiation-inhibiting polynucleotide are mammalian stem cells, e.g., HSCs from humans, mice, monkeys, farm animals, sport animals, pets, and other laboratory rodents and animals. These cells can be obtained, cultured, and manipulated as described above and in Potten C. S. ed., Stem Cells, Academic Press, 1997; Stem Cell Biology and Gene Therapy, eds. Quesenberry et al., John Wiley & Sons Inc., 1998; and Gage et al., Ann. Rev. Neurosci. 18:159-192, 1995.

Detail Description Paragraph:
[0065] As detailed in the Examples below, the present inventor identified a number of genes that are differentially expressed in human and mouse HSCs. These genes, which can play a role in regulating hematopoiesis as well as activities of HSCs and progenitor cells, are suitable as markers for selecting and enriching HSCs from diverse populations of cells. As exemplified in Tables 1-4, these HSC markers include transmembrane proteins (e.g., receptors), growth factor, transcription factors, as well as other proteins with diverse cellular and biochemical functions.

Detail Description Paragraph:
[0067] In some embodiments, the novel markers for selecting and enriching HSCs are cell surface markers. As described in the Examples, a number of the genes upregulated in the human and mouse HSCs encode transmembrane proteins (see also Tables 2 and 7). These proteins provide novel surface markers for isolating HSCs

from or enumerating HSCs in a population of diverse cells (e.g., bone marrow). These methods are useful for isolating stem cells from primates, e.g. human, monkeys, gorillas, domestic animals, bovine, equine, ovine, porcine, and etc. Isolation of HSCs bearing these novel markers can be performed with the same procedures disclosed herein for the other phenotypic markers.

Detail Description Paragraph:
[0070] For example, an initial crude cell population can be first purified to remove major cell families from the bone marrow or other hematopoietic cell source. A negative selection can then be carried out by targeting some of the cell surface antigens (e.g., Lin, CD34 for mouse HSCs). A further positive selection can be performed to isolate a cell population with specific stem cell markers (e.g., CD34 and Thy for human HSC, and c-kit, Sca-1, or CD38 for mouse HSC). Thereafter, additional selections can be carried out using one or more of the novel HSC surface markers disclosed herein.

Detail Description Paragraph:
[0078] Following the initial coarse selection, positive and/or negative selection using various other known stem cell markers as well as the novel HSC markers disclosed herein can be followed. In some methods, human HSCs are isolated using markers such as CD34.sup.+ and Thy.sup.+ as discussed in the Examples below. In some methods, human HSCs are selected for a phenotype of CD34.sup.+ Thy1.sup.+ Lin.sup.-. Other examples of enriched phenotypes include: CD2.sup.-, CD3.sup.-, CD4.sup.-, CD8.sup.-, CD10.sup.-, CD14.sup.-, CD15.sup.-, CD19.sup.-, CD20.sup.-, CD33.sup.-, CD34.sup.-, CD38.sup.lo/-, CD45RA.sup.-, CD 59.sup.+/-, CD71.sup.-, CDW109.sup.+, glycophorin.sup.-, AC133.sup.+, HLA.sup.-DR.sup.+/-, c-kit.sup.+, and EM.sup.+. Lin.sup.- refers to a cell population selected on the basis of lack of expression of at least one lineage specific marker, for example CD2, CD3, CD14, and CD56. The combination of expression markers used to isolate and define an enriched HSC population may vary depending on various factors and may vary as other expression markers become available.

Detail Description Paragraph:
[0079] Similarly, mouse HSCs can be selected for one or more of the known markers such as Lin.sup.-, c-kit.sup.+, Sca-1.sup.+, CD38.sup.+, and CD34.sup.- (see Example 3). In other methods, murine HSCs with similar properties to the human CD34.sup.+ Thy-1.sup.+ Lin.sup.- may be identified by kit+Thy-1.1.sup.lo Lin.sup.-/lo Sca-1.sup.+ (KTLS). Other phenotypes are well known, e.g., as described in U.S. Pat. No. 6,451,558. When CD34 expression is combined with selection for Thy-1, a composition comprising approximately fewer than 5% lineage committed cells can be isolated (U.S. Pat. No. 5,061,620).

Detail Description Paragraph:
[0081] Various culture media can be used and non-limiting examples include Iscove's modified Dulbecco's medium (IMDM), X-vivo 15 and RPMI-1640. These are commercially available from various vendors. The formulations may be supplemented with a variety of different nutrients, growth factors, such as cytokines and the like. In general, the term cytokine refers to any one of the numerous factors that exert a variety of effects on cells, such as inducing growth and proliferation. The cytokines may be human in origin or may be derived from other species when active on the cells of interest. Included within the scope of the definition are molecules having similar biological activity to wild type or purified cytokines, for example produced by recombinant means, and molecules which bind to a cytokine factor receptor and which elicit a similar cellular response as the native cytokine factor.

Detail Description Paragraph:
[0082] The medium can be serum free or supplemented with suitable amounts of serum such as fetal calf serum, autologous serum or plasma. If cells or cellular products are to be used in humans, the medium will preferably be serum free or supplemented with autologous serum or plasma (see, e.g., Lansdorp et al., J. Exp. Med. 175:1501,

1992; and Petzer et al., PNAS 93:1470, 1996).

Detail Description Paragraph:
[0088] The present invention provides methods for inhibiting HSC differentiation and promoting HSC expansion in vivo in a subject, e.g., a human subject engrafted with HSCs. Using HSC differentiation-inhibiting molecules identified in the present invention, these methods allow expansion of non-differentiated stem cells and increase the dose of HSCs either ex vivo or in vivo, thereby potentially allowing more rapid engraftment. The HSC differentiation-inhibiting molecules can be expressed in the engrafted HSCs. It can also be separately provided to the subject receiving the HSC graft, e.g., expressed from a vector introduced into the subject. In addition, the HSC differentiation-inhibit- ing molecules can also be administered to the subject as an expressed polypeptide, e.g., a growth factor. As a result, differentiation of the cells is blocked or slowed down, resulting in expansion of non-differentiated stem cells.

Detail Description Paragraph:
Genes Upregulated in Human HSCs

Detail Description Paragraph:
[0094] This Example describes RNA profiling of human hematopoietic stem cells and characterization of genes upregulated in the HSCs. All procedures and assays employed herein to study the human HSCs have been described in the art, e.g., as noted above.

Detail Description Paragraph:
[0095] CD34.sup.+ cells were first isolated from blood of six normal human donors using magnetic beads. Flow activated cell sorting (FACS) was then used to purify CD34.sup.+Thy.sup.+ (stem enriched) and CD34.sup.+Thy.sup.- (stem depleted) cell populations. The two populations of cells (total 12 samples, 6 CD34.sup.+Thy.sup.+ and 6 CD34.sup.+Thy.sup.-) were assayed for bioactivity with the CFC assay. RNA profiling (Thy.sup.+ vs Thy.sup.-) was then carried out to identify genes differentially expressed in stem cells. Results of the profiling are shown in Table 1. The data indicate that the upregulated genes encode proteins with diverse biochemical and cellular functions.

Detail Description Paragraph:
[0101] The HSC differentiation-inhibiting genes were also examined for their effects on HSC growth in liquid culture. The effect of GATA3 over-expression on human HSC differentiation was examined in liquid culture. Here, stem cells were transfected with the same vectors described above (which harbor the ID1 gene, GATA3 gene, or no HSC gene), and grown in liquid culture. CD34.sup.+ and GFP.sup.+ cells were sorted. Expression of CD34 was monitored during the culture. Cells without transfection were used in a control analysis. The results indicate that, as compared to the control, ID1 had no effect on differentiation of the CD34.sup.+ cells. However, expression of GATA3 significantly slowed the differentiation process as indicated by the rate of reduction of CD4.sup.+ cells.

Detail Description Paragraph:
[0106] Some of the differentially expressed genes were further analyzed and classified according to their biological functions. The results are shown in Table 6. As shown in Tables 3, 4, and 6, the upregulated genes in mouse HSCs also encode proteins of diverse biological properties, similar to genes upregulated in the human HSCs. For example, a number of transmembrane proteins were enriched in the mouse HSCs, as exemplified in Table 7. These molecules can be useful as novel surface markers for isolating HSCs. Some of transcription factors that are upregulated in the mouse HSCs are shown in Table 8. Their upregulated expression levels in the CD34.sup.-CD38.sup.+ HSCs relative to that in the facilitator cells (CD38.sup.-CD34.sup.+) and progenitor cells (CD34.sup.+CD38.sup.+) are shown in FIG. 3.

Detail Description Table CWU:
1TABLE 1 Genes upregulated in human CD34+Thy+ HSCs from peripheral blood Classification Name Description Histone H2BFL Homo sapiens H2B histone family, memberA Histone H2AFA Human histone genes Histone H2A/l Homo sapiens H2A histone family, member L Histone H1F2 Histone 2A-like protein gene Histone H2B/h Homo sapiens H2B histone family, member H Histone HH2A/c Human histone H2AFC gene Histone H2AFQ Homo sapiens H2A histone family, member Q HLA HLA-DPB1 Human MHC class II lymphocyte antigen beta chain HLA HLA-DQB1 Human MHC class II HLA-DR2-Dw12 mRNA DQw1-beta HLA HLA-E Homo sapiens HLA-E gene Secreted-complement PTS Homo sapiens 6-pyruvoyltetrahydroprotein synthase Secreted-complement HFL1 Human factor H homologue mRNA complete cds Secreted-growth MDK Homo sapiens midkine (neurite growth-promoting factor 2) factor Secreted-hormone OXT Homo sapiens oxytocin, prepro-(neurophysin 1) mRNA Secreted-hormone AVP Homo sapiens arginine vasopressin mRNA Signaling-GTP R-Ras Human R-ras Signaling-GTP GCHFR Homo sapiens GTP cyclohydrolase I feedback regulatory protein Signaling-GTP GUCY1A3 Homo sapiens guanylate cyclase 1, soluble, alpha 3 Signaling-Kinase WAF1 Human DNA sequence from PAC 431A14WAF1 Signaling-Kinase ITPKB Homo sapiens inositol 1,4,5-triphosphate 3-kinase B Signaling-Kinase PPKCL Homo sapiens protein kinase C, eta Signaling-Kinase PPKCZ Homo sapiens protein kinase C, zeta Signaling-SH3 SKAP55 Homo sapiens src kinase-associated phosphoprotein of 55 kDa Stress PTGS2 Homo sapiens prostaglandin-endoperoxide synthase 2 Stress CYP2A13 Human cytochrome P450 Stress CYP2D6 Human mRNA for cytochrome P450 dbl variant b Stress-apoptosis BCL2A1 Homo sapiens BCL-2-related protein 1 Structural CALB1 Homo sapiens calbindin 1 Structural Elastin Human elastin gene Structural KRT18 Human mRNA fragment for cytokeratin 18 Surface-Ig IGM Human gene for immunoglobulin mu Surface-Ig VH4 Human IgM heavy chain variable V-D-J region (VH4) gene Surface-other APP Homo sapiens APP complete sequence Surface-receptor BDKRB1 Human bradykinin B1 receptor Surface-receptor TLR1 Human mRNA for KIAA0012 gene Surface-receptor 5T4 Homo sapiens 5T4 oncofetal trophoblast glycoprotein Surface-receptor EFL-2 Homo sapiens EHK1 receptor tyrosine kinase ligand Surface-receptor EV12A Homo sapiens ecotropic viral integration site 2A Surface-receptor FLT3 Homo sapiens fms-related tyrosine kinase 3 Surface-receptor TNFSF10 Human tumor necrosis factor (ligand) superfamily, member 10 Surface-receptor LTB Human lymphotoxin beta Surface-receptor CDW52 Homo sapiens mRNA for CAMPATH-1 Surface-receptor CLECSF2 Homo sapiens C-type lectin (activation-induced) Surface-unknown GliPR Human glioma pathogenesis-related protein Transport LRP Homo sapiens Irp mRNA Transcription-RUNT AML1 Human AML1 protein Transcription-PAR-bZIP TEF Human hepatic leukemia factor Transcription-FKH FKHR Homo sapiens forkhead protein Transcription- MN1 Homo sapiens chromosome 22q11.2 MDR region suppressor Transcription-bHLH ID1 Homo sapiens inhibitor of DNA binding 1 Transcription-bHLH ID3 Homo sapiens HLH 1R21 mRNA for helix-loop-helix protein Transcription-bHLH EPAS1 Homo sapiens endothelial PAS domain protein 1 Transcription-bHLH ID2 Homo sapiens inhibitor of DNA binding 2 Transcription-GATA HGATA3 Homo sapiens GATA-binding protein 3 Transcription-HMG hTcf-4 Homo sapiens mRNA for hTCF-4 Transcription-HOX PHOX1 Human homeobox protein Transcription-HOX MEIS1 Homo sapiens MEIS protein Transcription- RBP-MS Homo sapiens RNA-binding protein gene with multiple slicing slicing Transcription- TCEA2 Homo sapiens transcription elongation factor A Translation Unknown DIF2 IEX-1 = radiation-inducible immediate-early gene Unknown Homo sapiens chromosome 17clone hRPC.906_A_24 Unknown Homo sapiens chromosome 22q13 BAC clone CIT987SK-384D8 Unknown A-362G6.1 Human chromosome 16 BAC clone CIT987SK-A-362G6 Unknown LST1 Homo sapiens LST1 mRNA Unknown KIAA0125 Homo sapiens KIAA0125 gene product

Detail Description Table CWU:
2TABLE 2 Genes Upregulated in Human HSCs from both Bone Marrow and Peripheral Blood Classification Name Description Hormone AVP Homo sapiens arginine vasopressin mRNA Hormone Corticotropin releasing hormone-binding protein Enzyme GUCY1A3 Homo sapiens guanylate cyclase 1, soluble, alpha 3 Enzyme PPKCZ Homo sapiens protein kinase C, zeta Enzyme Iduronate 2-sulfatase (Hunter syndrome) Transcription factor HLF Human hepatic leukemia factor Transcription factor GATA3 Homo sapiens GATA-binding

protein 3 Transcription Evil Homo sapiens ecotropic viral integration site 1 Transcription PMX1 Paired mesoderm homeo box 1 Transcription MN1 Meningioma (disrupted in balanced translocation) Secreted protein Tetranectin (plasminogen-binding protein) Secreted protein H factor (complement)-like 1 Surface molecule Transient receptor potential channel 1 Surface molecule DLK1 Delta-like homolog (Drosophila) Surface molecule EphA3 Ephrin-A3 Surface molecule TNFSF10 Human tumor necrosis factor (ligand) superfamily, member 10 Surface molecule Interferon induced transmembrane protein Surface molecule Ecotropic viral integration site 2A Surface molecule Sortilin-related receptor, L(DLR class) A rep Surface molecule Major histocompatibility complex, class I, E Surface molecule KIAA0125 gene product

Detail Description Table CWU:
Sfpq splicing factor proline/glutamine rich NM 023603 None 4.4 (polypyrimidine tract binding protein associated) 2010004A03Rik RIKEN cDNA 2010004A03 gene none None 4.3 Car2 carbonic anhydrase 2 NM 009801 Lyase Zinc 4.2 Mm.22896 ESTs NA None 4.1 AI573938 expressed sequence AI573938 none None 3.9 Vasp vasodilator-stimulated phosphoprotein none Actin-binding 3.9 Phosphorylation AA408451 expressed sequence AA408451 AA408451 None 3.7 Pftk1 PFTAIRE protein kinase 1 NM 011074 None 3.6 Tieg TGFB inducible early growth response NM 013692 DNA-binding Metal-binding 3.6 Nuclear protein Repeat Repressor Transcription regulation Zinc-finger Igk-V28 immunoglobulin kappa chain variable 28 (V28) none Immunoglobulin C region 3.6 Immunoglobulin domain Mm.1806 Mus musculus, Similar to KIAA1404 protein, NA None 3.5 clone IMAGE: 5252426, mRNA, partial cds Mm.25115 ESTs NA None 3.5 Ccrn41 CCR4 carbon catabolite repression 4-like none Biological rhythms 3.5 (S. cerevisiae) Cpo coproporphyrinogen oxidase NM 007757 Heine biosynthesis Iron 3.5 Mitochondrion Oxidoreductase Porphyrin biosynthesis Transit peptide Nupr1 nuclear protein 1 NM 019738 None 3.5 Mm.5510 similar to gene overexpressed in astrocytoma NA None 3.4 [Homo sapiens] Rab33b RAB33B, member of RAS oncogene family NM 016858 Golgi stack GTP-binding 3.4 Lipoprotein Prenylation Protein transport 9430065L19Rik RIKEN cDNA 9430065L19 gene NM 146083 None 3.4 Pgr progesterone receptor NM 008829 DNA-binding Nuclear 3.4 protein Receptor Steroid- binding Transcription regulation Zinc-finger LOC218490 similar to Transcription factor BTF3 (RNA NM 145455 Alternative splicing 3.4 polymerase B transcription factor 3) Nuclear protein Transcription regulation 4930434H03Rik RIKEN cDNA 4930434H03 gene none None 3.3 Actn3 Actinin alpha 3 NM 013456 Actin-binding Multigene 3.3 family Repeat Mm.202311 Mus musculus, clone IMAGE: 1379624, mRNA, NA GTP-binding Lipoprotein 3.3 partial cds Membrane Multigene family Palmitate Transducer Gtpi interferon-g induced GTPase NM 019440 None 3.3 Nat2 N-acetyltransferase 2 (arylamine N- NM 010874 Acyltransferase Multigene 3.3 acetyltransferase) family Polymorphism Transferase Eya2 eyes absent 2 homolog (Drosophila) none Alternative splicing 3.3 Developmental protein Multigene family 1110037N09Rik RIKEN cDNA 1110037N09 gene none None 3.2 5033414D02Rik RIKEN cDNA 5033414D02 gene NM 026362 None 3.1 Mm.26147 ESTs NA None 3.1 Il4 interleukin 4 NM 021283 B-cell activation Cytokine 3.1 Glycoprotein Growth factor Signal Ubap1 ubiquitin-associated protein 1 NM 023305 None 3.1 Acox1 acyl-Coenzyme A oxidase 1, palmitoyl NM 015729 FAD Fatty acid 2.9 metabolism Flavoprotein Oxidoreductase Peroxisome Ccl5 chemokine (C-C motif) ligand 5 NM 013653 Chemotaxis Cytokine 2.9 Inflammatory response Signal T-cell AW457192 expressed sequence AW457192 NM 134084 Cyclosporin Isomerase 2.9 Mitochondrion Multigene family Rotamase Transit peptide 2610016K11Rik RIKEN cDNA 2610016K11 gene none None 2.8 Fzd4 frizzled homolog 4 (Drosophila) NM 008055 Developmental protein 2.8 G-protein coupled receptor Glycoprotein Multigene family Signal Transmembrane Pla2g4a phospholipase A2, group IVA (cytosolic, NM 008869 Calcium Hydrolase Lipid 2.8 calcium-dependent) degradation Phosphorylation Scin scinderin NM 009132 None 2.7 NA AV239653 Mus musculus cDNA, 3 AV239653 None 2.7 end/clone = 4732435F04 /clone_end = 3/gb = AV239653 /gi = 6192160/ug = Mm.88313 /len = 214/NOTE = replacement for probe set(s) 96411_f_at on MG-U74A mRNA Tcf12 transcription factor 12 NM 011544 Alternative splicing 2.7 Developmental protein DNA-binding Nuclear protein Transcription regulation Madh7 MAD homolog 7 (Drosophila) NM 008543 Alternative splicing 2.7 Multigene family Transcription regulation Gem GTP binding protein (gene NM 010276 GTP-binding Membrane 2.7 overexpressed in skeletal muscle) Phosphorylation Tpm1

tropomyosin 1, alpha NM 024427 3D-structure Acetylation 2.7 Alternative splicing Coiled coil Multigene family Muscle protein Phosphorylation Repeat Map17 membrane-associated protein 17 NM 026018 None 2.7 Dcx doublecortin NM 010025 Neurogenesis Neurone 2.7 Phosphorylation Repeat Igk-V28 immunoglobulin kappa chain variable 28 (V28) none Immunoglobulin C region 2.6 Immunoglobulin domain Rnf11 ring finger protein 11 NM 013876 None 2.6 Nfix nuclear factor I/X NM 010906 None 2.6 Lin7c lin 7 homolog c (C. elegans) NM 011699 None 2.5 Cln3 ceroid lipofuscinosis, neuronal 3, juvenile NM 009907 Glycoprotein Lysosome 2.5 (Batten, Spielmeyer-Vogt disease) Transmembrane Hhex hematopoietically expressed homeobox NM 008245 Developmental protein 2.5 DNA-binding Homeobox Nuclear protein Gab1 growth factor receptor bound protein NM 021356 None 2.5 2-associated protein 1 None none none None 2.5 Kcnj3 potassium inwardly-rectifying channel, NM 008426 Ion transport Ionic channel 2.5 subfamily J, member 3 Potassium transport Transmembrane Voltage- gated channel Cradd CASP2 and RIPK1 domain containing adaptor NM 009950 Apoptosis 2.5 with death domain Mm.29914 ESTs NA None 2.4 Fos FBJ osteosarcoma oncogene NM 010234 DNA-binding Nuclear 2.4 protein Phosphorylation Proto-oncogene Mm.24247 ESTs NA None 2.4 4930472G13Rik RIKEN cDNA 4930472G13 gene NM 029447 None 2.4 Ormdl3 ORM1-like 3 (S. cerevisiae) NM 025661 None 2.4 Umpk uridine monophosphate kinase none Kinase Transferase 2.4 Creg cellular repressor of E1A-stimulated genes NM 011804 None 2.4 Utrn utrophin none None 2.3 Mm.27769 ESTs, Weakly similar to RIKEN cDNA 0610011E17 NA None 2.3 [Mus musculus] [M. musculus] Igtp interferon gamma induced GTPase NM 018738 None 2.3 Arg2 arginase type II NM 009705 Arginine metabolism 2.3 Hydrolase Manganese Mitochondrion Transit peptide Urea cycle Pklr pyruvate kinase liver and red blood NM 013631 Alternative splicing 2.2 cell Glycolysis Kinase Magnesium Multigene family Phosphorylation Transferase 1810010A06Rik RIKEN cDNA 1810010A06 gene NM 026921 None 2.2 Mm.532 ESTs, Weakly similar to lysophospholipase 1; NA None 2.2 phospholipase 1a; lysophopholipase 1 [Mus musculus] [M. musculus] Vamp5 vesicle-associated membrane protein 5 NM 016872 Multigene family 2.2 Myogenesis Signal-anchor Transmembrane 0710001O03Rik RIKEN cDNA 0710001O03 gene NM 146094 None 2.2 2610003J05Rik RIKEN cDNA 2610003J05 gene none None 2.2 Tdel1 tumor differentially expressed 1, like NM 019760 None 2.2 Serpinf1 serine (or cysteine) proteinase inhibitor, NM 011340 Glycoprotein Serpin Signal 2.1 clade F), member 1 Scotin scotin gene NM 025858 None 2.1 G3bp2 Ras-GTPase-activating protein (GAP<120>) NM 011816 None 2.1 SH3-domain binding protein 2 1190002H23Rik RIKEN cDNA 1190002H23 gene NM 025427 None 2.1 Nsccn1 non-selective cation channel 1 NM 010940 None 2.1 Tgoln2 trans-golgi network protein 2 NM 009444 None 2.1 Ywhae tyrosine 3-monooxygenase/tryptophan NM 009536 None 2.1 5-monooxygenase activation protein, epsilon polypeptide 4631408O11Rik RIKEN cDNA 4631408O11 gene none None 2.1 Pou2af1 POU domain, class 2, associating factor 1 NM 011136 Nuclear protein 2.1 Transcription regulation Mm.220953 Mus musculus, clone IMAGE: 4206769, NA None 2.1 mRNA

Detail Description Table CWU:

8TABLE 7 Tansmembrane Proteins Enriched in Mouse HSCs Classification Description surface Histocompatibility 2, class II antigen antigen E beta receptor Gamma-aminobutyric acid (GABA) B receptor, 1 oncogene Myeloproliferative leukemia virus oncogene (TPOR) surface Histocompatibility 2, class II antigen antigen A alpha Cytotoxic T lymphocyte-associated protein 2 beta receptor Erythropoietin receptor oncogene Kit oncogene Coagulation factor II (thrombin) receptor Frizzled homolog 4 (Drosophila) Membrane-associated protein 17 surface ESTs similar to C211_Human putative glycoprotein surface glycoprotein

CLAIMS:

3. The method of claim 1, wherein the stem cells are human hematopoietic stem cells.

12. The method of claim 8, wherein the subject is human, and the hematopoietic stem cells are human hematopoietic stem cells.

18. The method of claim 15, wherein the subject is human, and the HSC differentiation-inhibiting polypeptide is selected from the group shown in Table 2.

19. A method for isolating a population of cells that are enriched for hematopoietic stem cells (HSCs), the method comprising (a) obtaining a sample of cells containing hematopoietic stem cells, (b) selecting cells from the sample based on expression or lack of expression of at least one known HSC surface marker, and at least one molecule shown in Table 2 and Table 7 and (c) separating cells with the known HSC marker and at least one of the molecules shown in Table 2 and Table 7 thereby isolating a population of human cells enriched for hematopoietic stem cells.

20. The method of claim 19, wherein the hematopoietic stem cells are human HSCs.

29. The method of claim 27, wherein hematopoietic stem cells are human HSCs, and the population of cells are first selected for expression of CD34 and Thy prior to the contacting.

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L4: Entry 2 of 10 File: PGPB Oct 21, 2004

DOCUMENT-IDENTIFIER: US 20040210950 A1
TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (CALSARCINS)

Summary of Invention Paragraph:
[0006] Recent studies of calcineurin signaling in striated myocytes of heart and skeletal muscle have expanded the scope of important physiological and pathological events controlled by this ubiquitously expressed protein. Forced expression of a constitutively active form of calcineurin in hearts of transgenic mice promotes cardiac hypertrophy that progresses to dilated cardiomyopathy, heart failure, and death, in a manner that recapitulates features of human disease (Molkentin et al., 1998, herein incorporated by reference). Moreover, hypertrophy and heart failure in these animals, and in certain other animal models of cardiomyopathy, are prevented by administration of the calcineurin antagonist drugs cyclosporin A or FK-506 (Sussman et al., 1998). In skeletal muscles, calcineurin signaling is implicated both in hypertrophic growth stimulated by insulin-like growth factor-1 (Musaro et al., 1999; Semsarian et al., 1999), and in the control of specialized programs of gene expression that establish distinctive myofiber subtypes (Chin et al., 1998; Dunn et al., 1999). These observations have stimulated interest in the therapeutic potential of modifying calcineurin activity selectively in muscle cells while avoiding unwanted consequences of altered calcineurin signaling in other cell types (Sigal et al., 1991).

Summary of Invention Paragraph:
[0010] Drosophila .alpha.-actinin gene mutants are lethal, although the flies are able to survive beyond embryogenesis with detectable muscle dysfunction present at the hatching stage (Fyrberg et al., 1998). In larval development, the mutation manifests through noticeable muscle degeneration which progressively limits

mobility, and ultimately leads to death. Microscopic evaluation of mutant muscle fibers indicates that in as early as one-day old larvae, myofibrils are significantly perturbed with similar cellular pathologies to human nemaline myopathies.

Summary of Invention Paragraph:
[0018] In an additional embodiment of the present invention, there is a knockout non-human animal comprising a defective allele of a nucleic acid encoding calsarcin. In a specific embodiment, the animal further comprises two defective alleles of a nucleic acid encoding calsarcin. In an additional specific embodiment, the animal is a mouse.

Summary of Invention Paragraph:
[0019] In an additional embodiment of the present invention, there is a transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells. In specific embodiments, the promoter is constitutive, tissue specific, or inducible. In another specific embodiment the animal is a mouse.

Summary of Invention Paragraph:
[0030] In an additional embodiment of the present invention, there is a method of inhibiting calcineurin activation of gene transcription in a cell comprising providing to said cell a fusion protein comprising calsarcin, or a calcineurin-binding fragment thereof, fused to a targeting peptide that localizes said fusion protein to a subcellular region other than a subcellular region of normal function. In a specific embodiment, a targeting peptide comprises a geranylgeranyl group, a nuclear localization signal, a myristilation signal, and an endoplasmic reticulum signal peptide. In another specific embodiment, a cell is located in an animal. In a further specific embodiment, the animal is a human. In an additional specific embodiment the method further comprises treating said animal with a compound selected from the group consisting of an ionotrope, a beta blocker, an antiarrhythmic, a diuretic, a vasodilator, a hormone antagonist, an endothelin antagonist, an angiotensin type 2 antagonist and a cytokine inhibitor/blocker.

Summary of Invention Paragraph:
[0032] In an additional embodiment of the present invention, there is a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity comprising the steps of providing a cell lacking a functional calsarcin polypeptide; contacting said cell with said candidate substance; and determining the effect of said candidate substance on said cell. In a specific embodiment, the cell is a muscle cell. In another specific embodiment, the cell has a mutation in a regulatory region of calsarcin. In a further specific embodiment the mutation is a deletion mutation, an insertion mutation, or a point mutation. In a specific embodiment, the cell has a mutation in the coding region of calsarcin. In another specific embodiment, the mutation is a deletion mutation, an insertion mutation, a frameshift mutation, a nonsense mutation, a missense mutation or a splicing mutation. In further specific embodiments, the cell is contacted in vitro or in vivo. In an additional specific embodiment, the cell is located in a non-human transgenic animal

Brief Description of Drawings Paragraph:
[0034] FIGS. 1A-1E--Predicted amino acid sequences of human and mouse calsarcin-1 and calsarcin-2. The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E).

Brief Description of Drawings Paragraph:
[0035] FIGS. 2A-D--Nucleotide sequences for human calsarcin-1 (FIG. 2A), mouse

calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D).

Brief Description of Drawings Paragraph:
[0036] FIG. 3--Northern blot analysis of calsarcin-1 and calsarcin-2 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-1 mRNA is predominantly detected in heart and skeletal muscle, whereas the calsarcin-2 transcript was detected in skeletal muscle of both species.

Brief Description of Drawings Paragraph:
[0042] FIG. 9--Northern blot analysis of calsarcin-3 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-3 mRNA is predominantly detected in skeletal muscle, of both species.

Detail Description Paragraph:
[0049] Current results indicate that the interaction between calsarcin-1 and calcineurin is pertinent to the pathobiology, and ultimately to the therapy, of human heart disease. For example, familial forms of hypertrophic cardiomyopathy are caused by mutations in genes encoding proteins of the sarcomere (Seidman and Seidman, 1998) in a manner that likely involves calcineurin signaling (Marban et al., 1987). Administration of the calcineurin antagonist drugs cyclosporin A or FK-506 prevents cardiac hypertrophy in transgenic animal models of familial forms of hypertrophic cardiomyopathy (Sussman et al., 1998), but the analogous clinical trials are precluded because of toxic side effects (e.g., immunosuppression and hypertension) of existing agents.

Detail Description Paragraph:
[0050] Calcineurin antagonists also prevent cardiac hypertrophy and heart failure in some, although not all, animal models of acquired forms of cardiomyopathy that are common in human populations (Sussman et al., 1998; Ding et al., 1999; Zhang et al., 1999), but the same limitations to clinical trials apply. The relative abundance of calsarcin-1 in cardiac muscle makes it a prime target for drug development to circumvent these limitations of current calcineurin antagonists.

Detail Description Paragraph:
[0052] The significance of calcineurin-associated proteins in cardiomyopathies and muscular dystrophies is further indicated in the current invention. Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcins are likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0054] Applicants provide herein protein sequences for human calsarcin-1 (SEQ ID NO:2) and mouse calsarcin-1 (SEQ ID NO:4), human calsarcin-2 (SEQ ID NO:6), mouse calsarcin-2 (SEQ ID NO:8), human calsarcin-3 (SEQ ID NO: 10) and mouse calsarcin-3 (SEQ ID NO: 12). In a specific embodiment, a calcineurin associated sarcomeric protein (calsarcin) peptide, a calsarcin polypeptide or a calsarcin protein refer to calsarcin-1, calsarcin-2 or calsarcin-3. In addition to the entire calsarcin-1 molecules, the present invention also relates to fragments of the polypeptides that may or may not retain various of the functions described below. Fragments, including the N-terminus of the molecule, may be generated by genetic engineering

of translation stop sites within the coding region (discussed below). Alternatively, treatment of calsarcin-1 with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, 10, and 12, of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200 or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (e.g., ammonium sulfate), BPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

Detail Description Paragraph:
[0071] As described in the examples, the present inventors isolated calsarcin. Given the homology between human, mouse and rat calsarcin, determined by standard means in the art, an interesting series of mutants can be created by substituting homologous regions of various proteins. This is known, in certain contexts, as "domain switching."

Detail Description Paragraph:
[0093] The present invention also provides, in another embodiment, nucleic acids encoding calsarcin. Calsarcin nucleic acids include human calsarcin-1, human calsarcin-2, human calsarcin-3, mouse calsarcin-1, mouse calsarcin-2, and mouse calsarcin-3. Nucleic acids for human calsarcin-1 (SEQ ID NO: 1) and mouse calsarcin-1(SEQ ID NO:3) have been identified. In addition, three mouse calsarcin-2 ESTs and four human calsarcin-2 ESTs were identified (see Example 1). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945. In a specific embodiment, the mouse calsarcin-2 ESTs and the human calsarcin-2 ESTs are aligned by computer programs known in the art to identify full-length mouse calsarcin-2 and human calsarcin-2 sequences respectively. The present invention is not limited in scope to these nucleic acids. However, one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in various other species (e.g., rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

Detail Description Paragraph:
[0094] In another specific embodiment, calsarcin-3 was discovered "in silico" by comparing calsarcin I and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified. The full-length nucleic acid sequences from cDNA and genomic libraries are compared to differentiate between exon and intron sequences (Sambrook, et al, 1989). Furthermore, computer programs well known in the art use the nucleic acid sequence to generate a predicted amino acid sequence.

Detail Description Paragraph:
[0095] In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a "calsarcin nucleic acid" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the human and mouse nucleic acids disclosed herein.

Detail Description Paragraph:
[0106] Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers

are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,20,25,30,35,40,45, 50, 55, 60,65, 70, 75, 80, 85,90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, or 3000 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

Detail Description Paragraph:
[0116] Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Detail Description Paragraph:
[0132] In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Detail Description Paragraph:
[0139] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detail Description Paragraph:
[0154] The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Detail Description Paragraph:
[0157] Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1,

the D3 or both regions (Graham and Prevac, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Detail Description Paragraph:
[0158] Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detail Description Paragraph:
[0160] Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detail Description Paragraph:
[0167] A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al, 1989).

Detail Description Paragraph:
[0213] Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Detail Description Paragraph:
[0226] To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detail Description Paragraph:
[0236] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks and drugs--in

a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Detail Description Paragraph:
[0237] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Detail Description Paragraph:
[0245] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in I ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Detail Description Paragraph:
[0253] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detail Description Paragraph:
[0278] Thus, in accordance with the present invention there is provided herein a method of screening for a candidate substance for anti-cardiomyopic hypertrophy

activity or anti-heart failure activity by providing a cell lacking a functional calsarcin polypeptide, contacting the cell with a candidate substance and determining the effect of the candidate substance on the cell. The cell lacking a functional calsarcin polypeptide is described elsewhere herein and may derive from a transgenic non-human animal containing the cell, as in a cell line. The cell is preferably a muscle cell and may have a mutation in a regulatory region of calsarcin, such as a deletion, insertion or point mutation, or in the coding region, such as a deletion, insertion, frameshift, nonsense, missense or splicing mutation. The cell may be contacted in vitro or in vivo by methods well known in the art, and in a specific embodiment is located in a non-human transgenic animal.

Detail Description Paragraph:
[0293] Yeast Two-Hybrid Screens. A full-legnth mouse CnA-.alpha. cDNA, fused to the GAL4 DNA binding domain was used as bait in a two-hybrid screen of approximately 1.5.times.10.sup.6 clones of a human heart cDNA library (Clontech), as described previously (Molkentin et al., 1998). From this screen, the inventors identified a cDNA encoding calsarcin-1. Additional two-hybrid screens of the same cDNA library were performed using calsarcin-1 and calsarcin-2 as bait.

Detail Description Paragraph:
[0294] Northern blot analysis. Northern blots of RNA from human and mouse multiple tissues (Clontech) as well as from C2C12 cell extracts were performed as described (Spencer et al., 2000).

Detail Description Paragraph:
[0301] Searching public expressed sequence tag (EST) databases with the mouse calsarcin-1 cDNA sequence, human calsarcin-1 cDNA clones were identified, as well as human and mouse sequences for the related genes calsarcin-2 and calsarcin-3. A skilled artisan is aware of databases available for such searching of both protein and nucleic acid sequences, including GenBank (http://www.ncbi.nim.nih.gov/Genbank/GenbankSearch.html-) or commercially available databases (Celera Genomics, Inc.; Rockville, Md.; www.celera.com). Alignment of calsarcins 1-3 is demonstrated in FIG. 13.

Detail Description Paragraph:
[0302] The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E). Also provided are DNA sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D). Calsarcin-1 and -2 show the highest homology toward their amino- and carboxy-termini, whereas the intervening amino acids are less well conserved. BLATS searches with both proteins sequences did not reveal any significant homology to know proteins.

Detail Description Paragraph:
[0303] Calsarcin-2 was identified by searching the EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) with the sequence of calsarcin-1. Three mouse calsarcin-2 ESTs were identified: GenBank accession numbers (AA036142, AW742494, W29466). Additionally, four human calsarcin-2 ESTs were identified: GenBank accession numbers (AW964108, AA197193, AW000988, AA176945). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945.

Detail Description Paragraph:
[0304] Calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a

mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified.

Detail Description Paragraph:
[0312] To determine which tissues calsarcin-1 and calsarcin-2 are expressed in, Northern analysis was performed. In FIG. 3, polyA+ RNA from the indicated mouse tissues was analyzed for expression of calsarcin-1 and calsarcin-2 transcripts by methods well known in the art. The data shows a highly striated, muscle-specific expression pattern for calsarcin-1 and -2. Calsarcin-1 is specifically expressed in the heart and skeletal muscle, with two mRNAs of 1.6 and 2.6 kb in human tissues, and only a single transcript of 1.3 kb in mouse. Faint expression of calsarcin-1 was also detected in mouse lung and liver. A 1.6 kb and 1.3 kb calsarcin-2 transcript was detected exclusively in adult human and mouse skeletal muscle, respectively. The relative difference in expression level of calsarcin-1 between human and mouse skeletal muscle may reflect differences in slow- versus fast-twitch fiber composition.

Detail Description Paragraph:
[0324] N- and C-terminal truncations of calsarcin-1 were used to characterize the CnA and .alpha.-actinin interaction domains. Yeast two-hybrid assays and complementary immunoprecipitation experiments revealed that amino acids 153-200 are necessary for interaction of calsarcin with .alpha.-actinin-2 (FIG. 7). Twenty-five residues within this region are highly conserved between mouse and human calsarcin-1 and -2, suggesting that this might constitute the minimal interaction domain. Since a motif between amino acids 245-250 resembles known calcineurin dockings sites on NFAT (PxIxIT) and MCIP (PxIxxIT), the inventors tested a C-terminai truncation lacking both those residues. However, calsarcin lacking these amino acids was still able to bind can, both by two-hybrid assay (GAL4-calsarcin 85-240) and coimmunoprecipitation (Myg-calsarcin 1-240). In contrast, a calsarcin-I mutant lacking residues 217-264 was unable to bind CnA, implying that residues 217-240 are necessary for binding. Mapping of the interaction domain on CnA revealed that the calsarcin-interacting domain residues within the catalytic region, whereas the calsarcin-1 interacting domain of .alpha.-actinin maps to the second and third spectrin-like repeats.

Detail Description Paragraph:
[0326] Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcin-1 is likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0345] Barski O A, Gabbay K H, Bohren K M. Characterization of the human aldehyde reductase gene and promoter. Genomics 60(2):188-98 (1999).

Detail Description Paragraph:
[0346] Beggs A H, Byers T J, Knoll J H, Boyce F M, Bruns G A, Kunkel L M. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J Biol Chem. May 5;267(13):9281-8 (1992).

Detail Description Paragraph:
[0348] Bhavsar P K, Brand N J, Yacoub M H, Barton P J R. Isolation and characterization of the human cardiac troponin I gene (TNN13).Genomics. July 1;35 (1):11-23 (1996).

Detail Description Paragraph:
[0375] Landon F, Gache Y, Touitou H, Olomucki A. Properties of two isoforms of human blood platelet -alpha-actinin. Eur J Biochem. December 2;153(2):231-7 (1985).

Detail Description Paragraph:
[0376] LaPointe M C, Wu G, Garami M, Yang X P, Gardner D G. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes. Hypertension. March;27(3 Pt 2):715-22 (1996).

Detail Description Paragraph:
[0396] Ritchie M E. Characterization of human B creatine kinase gene regulation in the heart in vitro and in vivo. J Biol Chem. October 11;271(41):25485-91 (1996).

Detail Description Table CWU:
2TABLE 2 Promoter and/or Enhancer Promoter/Enhancer References Immunoglobulin Heavy Chain Banerji et al., 1983; Gilles et al., 1983; Grosschedl et al., 1985; Atchinson et al., 1986, 1987; Imler et al., 1987; Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al.; 1990 Immunoglobulin Light Chain Queen et al., 1983; Picard et al., 1984 T-Cell Receptor Luria et al., 1987; Winoto et al., 1989; Redondo et al.; 1990 HLA DQ a and/or DQ Sullivan et al., 1987 -Interferon Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988 Interleukin-2 Greene et al., 1989 Interleukin-2 Receptor Greene et al., 1989; Lin et al., 1990 MHC Class II 5 Koch et al., 1989 MHC Class II HLA-DRa Sherman et al., 1989 -Actin Kawamoto et al., 1988; Ng et al.; 1989 Muscle Creatine Kinase (MCK) Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., 1989 Prealbumin (Transthyretin) Costa et al., 1988 Elastase I Ornitz et al., 1987 Metallothionein (MTII) Karin et al., 1987; Culotta et al., 1989 Collagenase Pinkert et al., 1987; Angel et al., 1987a Albumin Pinkert et al., 1987; Tronche et al., 1989, 1990 -Fetoprotein Godbout et al., 1988; Campere et al., 1989 t-Globin Bodine et al., 1987; Perez-Stable et al., 1990 -Globin Trudel et al., 1987 c-fos Cohen et al., 1987 c-HA-ras Triesman, 1986; Deschamps et al., 1985 Insulin Edlund et al., 1985 Neural Cell Adhesion Molecule Hirsh et al., 1990 (NCAM) .sub.1-Antitrypain Latimer et al., 1990 H2B (TH2B) Histone Hwang et al., 1990 Mouse and/or Type I Collagen Ripe et al., 1989 Glucose-Regulated Proteins Chang et al., 1989 (GRP94 and GRP78) Rat Growth Hormone Larsen et al., 1986 Human Serum Amyloid A (SAA) Edbrooke et al., 1989 Troponin I (TN I) Yutzey et al., 1989 Platelet-Derived Growth Factor Pech et al., 1989 (PDGF) Duchenne Muscular Dystrophy Klamut et al., 1990 SV40 Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Firak et al., 1986; Herr et al., 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988 Polyoma Swartzendruber et al., 1975; Vasseur et al., 1980; Katinka et al., 1980, 1981; Tyndell et al., 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and/or Villarreal, 1988 Retroviruses Kriegler et al., 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander et al., 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman et al., 1989 Papilloma Virus Campo et al., 1983; Lusky et al., 1983; Spandidos and/or Wilkie, 1983; Spalholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens et al., 1987; Glue et al., 1988 Hepatitis B Virus Bulla et al., 1986; Jameel et al., 1986; Shaul et al., 1987; Spandau et al., 1988; Vannice et al., 1988 Human Immunodeficiency Virus Muesing et al., 1987; Hauber et al., 1988; Jakobovits et al., 1988; Feng et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp et al., 1989; Braddock et al., 1989 Cytomegalovirus (CMV) Weber et al., 1984; Boshart et al., 1985; Foecking et al., 1986 Gibbon Ape Leukemia Virus Holbrook et al., 1987; Quinn et al., 1989

CLAIMS:

22. A knockout non-human animal comprising a defective allele of a nucleic acid encoding a calcineurin associated sarcomeric protein (calsarcin).

25. A transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells.

93. The method of claim 92 wherein said animal is a human.

105. The method of claim 105, wherein said cell is located in a non-human transgenic animal.

☐ 3. Document ID: US 20040186275 A1

L4: Entry 3 of 10 File: PGPB Sep 23, 2004

DOCUMENT-IDENTIFIER: US 20040186275 A1
TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

Summary of Invention Paragraph:
[0007] Recent studies of calcineurin signaling in striated myocytes of heart and skeletal muscle have expanded the scope of important physiological and pathological events controlled by this ubiquitously expressed protein. Forced expression of a constitutively active form of calcineurin in hearts of transgenic mice promotes cardiac hypertrophy that progresses to dilated cardiomyopathy, heart failure, and death, in a manner that recapitulates features of human disease (Molkentin et al, 1998, herein incorporated by reference). Moreover, hypertrophy and heart failure in these animals, and in certain other animal models of cardiomyopathy, are prevented by administration of the calcineurin antagonist drugs cyclosporin A or FK-506 (Sussman et al., 1998). In skeletal muscles, calcineurin signaling is implicated both in hypertrophic growth stimulated by insulin-like growth factor-1 (Musaro et al., 1999; Semsarian et al., 1999), and in the control of specialized programs of gene expression that establish distinctive myofiber subtypes (Chin et al., 1998; Dunn et al., 1999). These observations have stimulated interest in the therapeutic potential of modifying calcineurin activity selectively in muscle cells while avoiding unwanted consequences of altered calcineurin signaling in other cell types (Sigal et al., 1991).

Summary of Invention Paragraph:
[0011] Drosophila .alpha.-actinin gene mutants are lethal, although the flies are able to survive beyond embryogenesis with detectable muscle dysfunction present at the hatching stage (Fyrberg et al., 1998). In larval development, the mutation manifests through noticeable muscle degeneration which progressively limits mobility, and ultimately leads to death. Microscopic evaluation of mutant muscle fibers indicates that in as early as one-day old larvae, myofibrils are significantly perturbed with similar cellular pathologies to human nemaline myopathies.

Summary of Invention Paragraph:
[0019] In an additional embodiment of the present invention, there is a knockout non-human animal comprising a defective allele of a nucleic acid encoding calsarcin. In a specific embodiment, the animal further comprises two defective

alleles of a nucleic acid encoding calsarcin. In an additional specific embodiment, the animal is a mouse.

Summary of Invention Paragraph:
[0020] In an additional embodiment of the present invention, there is a transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells. In specific embodiments, the promoter is constitutive, tissue specific, or inducible. In another specific embodiment the animal is a mouse.

Summary of Invention Paragraph:
[0032] In an additional embodiment of the present invention, there is a method of inhibiting calcineurin activation of gene transcription in a cell comprising providing to said cell a fusion protein comprising calsarcin, or a calcineurin-binding fragment thereof, fused to a targeting peptide that localizes said fusion protein to a subcellular region other than a subcellular region of normal function. In a specific embodiment, a targeting peptide comprises a geranylgeranyl group, a nuclear localization signal, a myristilation signal, and an endoplasmic reticulum signal peptide. In another specific embodiment, a cell is located in an animal. In a further specific embodiment, the animal is a human. In an additional specific embodiment the method further comprises treating said animal with a compound selected from the group consisting of an ionotrope, a beta blocker, an antiarrhythmic, a diuretic, a vasodilator, a hormone antagonist, an endothelin antagonist, an angiotensin type 2 antagonist and a cytokine inhibitor/blocker.

Summary of Invention Paragraph:
[0034] In an additional embodiment of the present invention, there is a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity comprising the steps of providing a cell lacking a functional calsarcin polypeptide; contacting said cell with said candidate substance; and determining the effect of said candidate substance on said cell. In a specific embodiment, the cell is a muscle cell. In another specific embodiment, the cell has a mutation in a regulatory region of calsarcin. In a further specific embodiment the mutation is a deletion mutation, an insertion mutation, or a point mutation. In a specific embodiment, the cell has a mutation in the coding region of calsarcin. In another specific embodiment, the mutation is a deletion mutation, an insertion mutation, a frameshift mutation, a nonsense mutation, a missense mutation or a splicing mutation. In further specific embodiments, the cell is contacted in vitro or in vivo. In an additional specific embodiment, the cell is located in a non-human transgenic animal

Brief Description of Drawings Paragraph:
[0036] FIGS. 1A-1E--Predicted amino acid sequences of human and mouse calsarcin-1 and calsarcin-2. The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E).

Brief Description of Drawings Paragraph:
[0037] FIGS. 2A-D--Nucleotide sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D).

Brief Description of Drawings Paragraph:
[0038] FIG. 3--Northern blot analysis of calsarcin-1 and calsarcin-2 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-1 mRNA is predominantly detected in heart and skeletal muscle, whereas the calsarcin-2 transcript was detected in skeletal muscle of both species.

Brief Description of Drawings Paragraph:
[0044] FIG. 9--Northern blot analysis of calsarcin-3 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-3 mRNA is predominantly detected in skeletal muscle, of both species.

Detail Description Paragraph:
[0051] Current results indicate that the interaction between calsarcin-I and calcineurin is pertinent to the pathobiology, and ultimately to the therapy, of human heart disease. For example, familial forms of hypertrophic cardiomyopathy are caused by mutations in genes encoding proteins of the sarcomere (Seidman and Seidman, 1998) in a manner that likely involves calcineurin signaling (Marban et al., 1987). Administration of the calcineurin antagonist drugs cyclosporin A or FK-506 prevents cardiac hypertrophy in transgenic animal models of familial forms of hypertrophic cardiomyopathy (Sussman et al., 1998), but the analogous clinical trials are precluded because of toxic side effects (e.g., immunosuppression and hypertension) of existing agents.

Detail Description Paragraph:
[0052] Calcineurin antagonists also prevent cardiac hypertrophy and heart failure in some, although not all, animal models of acquired forms of cardiomyopathy that are common in human populations (Sussman et al., 1998; Ding et al., 1999; Zhang et al, 1999), but the same limitations to clinical trials apply. The relative abundance of calsarcin-1 in cardiac muscle makes it a prime target for drug development to circumvent these limitations of current calcineurin antagonists.

Detail Description Paragraph:
[0054] The significance of calcineurin-associated proteins in cardiomyopathies and muscular dystrophies is further indicated in the current invention. Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcins are likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0056] Applicants provide herein protein sequences for human calsarcin-1 (SEQ ID NO:2) and mouse calsarcin-1 (SEQ ID NO:4), human calsarcin-2 (SEQ ID NO:6), mouse calsarcin-2 (SEQ ID NO:8), human calsarcin-3 (SEQ ID NO:110) and mouse calsarcin-3 (SEQ ID NO: 12). In a specific embodiment, a calcineurin associated sarcomeric protein (calsarcin) peptide, a calsarcin polypeptide or a calsarcin protein refer to calsarcin-1, calsarcin-2 or calsarcin-3. In addition to the entire calsarcin-1 molecules, the present invention also relates to fragments of the polypeptides that may or may not retain various of the functions described below. Fragments, including the N-terminus of the molecule, may be generated by genetic engineering of translation stop sites within the coding region (discussed below). Alternatively, treatment of calsarcin-1 with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, 10, and 12, of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200 or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (e.g., ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size

separations (sedimentation, gel electrophoresis, gel filtration).

Detail Description Paragraph:
[0073] As described in the examples, the present inventors isolated calsarcin. Given the homology between human, mouse and rat calsarcin, determined by standard means in the art, an interesting series of mutants can be created by substituting homologous regions of various proteins. This is known, in certain contexts, as "domain switching."

Detail Description Paragraph:
[0095] The present invention also provides, in another embodiment, nucleic acids encoding calsarcin. Calsarcin nucleic acids include human calsarcin-1, human calsarcin-2, human calsarcin-3, mouse calsarcin-1, mouse calsarcin-2, and mouse calsarcin-3. Nucleic acids for human calsarcin-1 (SEQ ID NO:1) and mouse calsarcin-1 (SEQ ID NO:3) have been identified. In addition, three mouse calsarcin-2 ESTs and four human calsarcin-2 ESTs were identified (see Example 1). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945. In a specific embodiment, the mouse calsarcin-2 ESTs and the human calsarcin-2 ESTs are aligned by computer programs known in the art to identify full-length mouse calsarcin-2 and human calsarcin-2 sequences respectively. The present invention is not limited in scope to these nucleic acids. However, one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in various other species (e.g., rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

Detail Description Paragraph:
[0096] In another specific embodiment, calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified. The full-length nucleic acid sequences from cDNA and genomic libraries are compared to differentiate between exon and intron sequences (Sambrook, et al., 1989). Furthermore, computer programs well known in the art use the nucleic acid sequence to generate a predicted amino acid sequence.

Detail Description Paragraph:
[0097] In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a "calsarcin nucleic acid" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the human and mouse nucleic acids disclosed herein.

Detail Description Paragraph:
[0108] Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, or 3000 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in

amplification reactions.

Detail Description Paragraph:
[0118] Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Detail Description Paragraph:
[0134] In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Detail Description Paragraph:
[0141] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detail Description Paragraph:
[0156] The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Detail Description Paragraph:
[0159] Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by AdS DNA fragments and constitutively expresses E1 proteins (Graham et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevac, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of

infection (MOI) (Mulligan, 1993).

Detail Description Paragraph:
[0160] Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detail Description Paragraph:
[0162] Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detail Description Paragraph:
[0169] A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

Detail Description Paragraph:
[0215] Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Detail Description Paragraph:
[0228] To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detail Description Paragraph:
[0238] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks and drugs--in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Detail Description Paragraph:
[0239] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells,

dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Detail Description Paragraph:
[0247] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Detail Description Paragraph:
[0255] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detail Description Paragraph:
[0280] Thus, in accordance with the present invention there is provided herein a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity by providing a cell lacking a functional calsarcin polypeptide, contacting the cell with a candidate substance and determining the effect of the candidate substance on the cell. The cell lacking a functional calsarcin polypeptide is described elsewhere herein and may derive from a transgenic non-human animal containing the cell, as in a cell line. The cell is preferably a muscle cell and may have a mutation in a regulatory region of calsarcin, such as a deletion, insertion or point mutation, or in the coding region, such as a deletion, insertion, frameshift, nonsense, missense or splicing mutation. The cell may be contacted in vitro or in vivo by methods well known in

the art, and in a specific embodiment is located in a non-human transgenic animal.

Detail Description Paragraph:
[0295] Yeast Two-Hybrid Screens. A full-legnth mouse CnA-.alpha. cDNA, fused to the GAL4 DNA binding domain was used as bait in a two-hybrid screen of approximately 1.5.times.10.sup.6 clones of a human heart cDNA library (Clontech), as described previously (Molkentin et al., 1998). From this screen, the inventors identified a cDNA encoding calsarcin-1. Additional two-hybrid screens of the same cDNA library were performed using calsarcin-1 and calsarcin-2 as bait.

Detail Description Paragraph:
[0296] Northern blot analysis. Northern blots of RNA from human and mouse multiple tissues (Clontech) as well as from C2C12 cell extracts were performed as described (Spencer et al., 2000).

Detail Description Paragraph:
[0303] Searching public expressed sequence tag (EST) databases with the mouse calsarcin-1 cDNA sequence, human calsarcin-1 cDNA clones were identified, as well as human and mouse sequences for the related genes calsarcin-2 and calsarcin-3. A skilled artisan is aware of databases available for such searching of both protein and nucleic acid sequences, including GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html-) or commercially available databases (Celera Genomics, Inc.; Rockville, Md.; www.celera.com). Alignment of calsarcins 1-3 is demonstrated in FIG. 13.

Detail Description Paragraph:
[0304] The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E). Also provided are DNA sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D). Calsarcin-1 and -2 show the highest homology toward their amino- and carboxy-termini, whereas the intervening amino acids are less well conserved. BLATS searches with both proteins sequences did not reveal any significant homology to know proteins.

Detail Description Paragraph:
[0305] Calsarcin-2 was identified by searching the EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) with the sequence of calsarcin-1. Three mouse calsarcin-2 ESTs were identified: GenBank accession numbers (AA036142, AW742494, W29466). Additionally, four human calsarcin-2 ESTs were identified: GenBank accession numbers (AW964108, AA197193, AW000988, AA176945). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945.

Detail Description Paragraph:
[0306] Calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified.

Detail Description Paragraph:
[0314] To determine which tissues calsarcin-1 and calsarcin-2 are expressed in, Northern analysis was performed. In FIG. 3, polyA+ RNA from the indicated mouse tissues was analyzed for expression of calsarcin-1 and calsarcin-2 transcripts by methods well known in the art. The data shows a highly striated, muscle-specific expression pattern for calsarcin-1 and -2. Calsarcin-1 is specifically expressed in

the heart and skeletal muscle, with two mRNAs of 1.6 and 2.6 kb in human tissues, and only a single transcript of 1.3 kb in mouse. Faint expression of calsarcin-1 was also detected in mouse lung and liver. A 1.6 kb and 1.3 kb calsarcin-2 transcript was detected exclusively in adult human and mouse skeletal muscle, respectively. The relative difference in expression level of calsarcin-1 between human and mouse skeletal muscle may reflect differences in slow- versus fast-twitch fiber composition.

Detail Description Paragraph:
[0326] N- and C-terminal truncations of calsarcin-1 were used to characterize the CnA and .alpha.-actinin interaction domains. Yeast two-hybrid assays and complementary immunoprecipitation experiments revealed that amino acids 153-200 are necessary for interaction of calsarcin with .alpha.-actinin-2 (FIG. 7). Twenty-five residues within this region are highly conserved between mouse and human calsarcin-1 and -2, suggesting that this might constitute the minimal interaction domain. Since a motif between amino acids 245-250 resembles known calcineurin dockings sites on NFAT (PxIxIT) and MCIP (PxIxxfT), the inventors tested a C-terminal truncation lacking both those residues. However, calsarcin lacking these amino acids was still able to bind can, both by two-hybrid assay (GAL4-calsarcin 85-240) and coimmunoprecipitation (Myc-calsarcin 1-240). In contrast, a calsarcin-1 mutant lacking residues 217-264 was unable to bind CnA, implying that residues 217-240 are necessary for binding. Mapping of the interaction domain on CnA revealed that the calsarcin-interacting domain residues within the catalytic region, whereas the calsarcin-1 interacting domain of .alpha.-actinin maps to the second and third spectrin-like repeats.

Detail Description Paragraph:
[0328] Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcin-1 is likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0347] Barski O A, Gabbay K H, Bohren K M. Characterization of the human aldehyde reductase gene and promoter. Genomics 60(2):188-98 (1999).

Detail Description Paragraph:
[0348] Beggs A H, Byers T J, Knoll J H, Boyce F M, Bruns G A, Kunkel L M. Cloning and characterization of two human skeletal muscle alph.alpha.-actinin genes located on chromosomes 1 and 11. J Biol Chem. May 5;267(13):9281-8 (1992).

Detail Description Paragraph:
[0350] Bhavsar P K, Brand N J, Yacoub M H, Barton P J R. Isolation and characterization of the human cardiac troponin I gene (TNN13).Genomics. Jul 1;35 (1):11-23 (1996).

Detail Description Paragraph:
[0377] Landon F, Gache Y, Touitou H, Olomucki A. Properties of two isoforms of human blood platelet alph.alpha.-actinin. Eur J Biochem. Dec 2; 153(2):231-7 (1985).

Detail Description Paragraph:
[0378] LaPointe M C, Wu G, Garami M, Yang X P, Gardner D G. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes.

Hypertension. Mar;27(3 Pt 2):715-22 (1996).

Detail Description Paragraph:
[0399] Ritchie M E. Characterization of human B creatine kinase gene regulation in the heart in vitro and in vivo. J Biol Chem. Oct 11;271(41):25485-91 (1996).

Detail Description Table CWU:
2TABLE 2 Promoter and/or Enhancer Promoter/Enhancer References Immunoglobulin Heavy Chain Banerji et al., 1983; Gilles et al., 1983; Grosschedl et al., 1985; Atchinson et al., 1986, 1987; Imler et al., 1987; Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al.; 1990 Immunoglobulin Light Chain Queen et al., 1983; Picard et al., 1984 T-Cell Receptor Luria et al., 1987; Winoto et al., 1989; Redondo et al.; 1990 HLA DQ a and/or DQ Sullivan et al., 1987 Interferon Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988 Interleukin-2 Greene et al., 1989 Interleukin-2 Receptor Greene et al., 1989; Lin et al., 1990 MHC Class II 5 Koch et al., 1989 MHC Class II HLA-DRa Sherman et al., 1989 Actin Kawamoto et al., 1988; Ng et al.; 1989 Muscle Creatine Kinase (MCK) Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., 1989 Prealbumin (Transthyretin) Costa et al., 1988 Elastase I Ornitz et al., 1987 Metallothionein (MTII) Karin et al., 1987; Culotta et al., 1989 Collagenase Pinkert et al., 1987; Angel et al., 1987a Albumin Pinkert et al., 1987; Tronche et al., 1989, 1990 Fetoprotein Godbout et al., 1988; Campere et al., 1989 t-Globin Bodine et al., 1987; Perez-Stable et al., 1990 Globin Trudel et al., 1987 c-fos Cohen et al., 1987 c-HA-ras Triesman, 1986; Deschamps et al., 1985 Insulin Edlund et al., 1985 Neural Cell Adhesion Molecule Hirsh et al., 1990 (NCAM) 1-Antitrypain Latimer et al., 1990 H2B (TH2B) Histone Hwang et al., 1990 Mouse and/or Type I Collagen Ripe et al., 1989 Glucose-Regulated Proteins Chang et al., 1989 (GRP94 and GRP78) Rat Growth Hormone Larsen et al., 1986 Human Serum Amyloid A (SAA) Edbrooke et al., 1989 Troponin I (TN I) Yutzey et al., 1989 Platelet-Derived Growth Factor Pech et al., 1989 (PDGF) Duchenne Muscular Dystrophy Klamut et al., 1990 SV40 Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Firak et al., 1986; Herr et al., 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988 Polyoma Swartzendruber et al., 1975; Vasseur et al., 1980; Katinka et al., 1980, 1981; Tyndell et al., 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and/or Villarreal, 1988 Retroviruses Kriegler et al., 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander et al., 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman et al., 1989 Papilloma Virus Campo et al., 1983; Lusky et al., 1983; Spandidos and/or Wilkie, 1983; Spalholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens et al., 1987; Glue et al., 1988 Hepatitis B Virus Bulla et al., 1986; Jameel et al., 1986; Shaul et al., 1987; Spandau et al., 1988; Vannice et al., 1988 Human Immunodeficiency Virus Muesing et al., 1987; Hauber et al., 1988; Jakobovits et al., 1988; Feng et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp et al., 1989; Braddock et al., 1989 Cytomegalovirus (CMV) Weber et al., 1984; Boshart et al., 1985; Foecking et al., 1986 Gibbon Ape Leukemia Virus Holbrook et al., 1987; Quinn et al., 1989

CLAIMS:

22. A knockout non-human animal comprising a defective allele of a nucleic acid encoding a calcineurin associated sarcomeric protein (calsarcin).

25. A transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells.

93. The method of claim 92 wherein said animal is a human.

105. The method of claim 105, wherein said cell is located in a non-human transgenic animal.

☐ 4. Document ID: US 20040127686 A1

L4: Entry 4 of 10 File: PGPB Jul 1, 2004

DOCUMENT-IDENTIFIER: US 20040127686 A1
TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

Summary of Invention Paragraph:
[0006] Recent studies of calcineurin signaling in striated myocytes of heart and skeletal muscle have expanded the scope of important physiological and pathological events controlled by this ubiquitously expressed protein. Forced expression of a constitutively active form of calcineurin in hearts of transgenic mice promotes cardiac hypertrophy that progresses to dilated cardiomyopathy, heart failure, and death, in a manner that recapitulates features of human disease (Molkentin et al, 1998, herein incorporated by reference). Moreover, hypertrophy and heart failure in these animals, and in certain other animal models of cardiomyopathy, are prevented by administration of the calcineurin antagonist drugs cyclosporin A or FK-506 (Sussman et al, 1998). In skeletal muscles, calcineurin signaling is implicated both in hypertrophic growth stimulated by insulin-like growth factor-1 (Musaro et al, 1999; Semsarian et al, 1999), and in the control of specialized programs of gene expression that establish distinctive myofiber subtypes (Chin et al, 1998; Dunn et al, 1999). These observations have stimulated interest in the therapeutic potential of modifying calcineurin activity selectively in muscle cells while avoiding unwanted consequences of altered calcineurin signaling in other cell types (Sigal et al, 1991).

Summary of Invention Paragraph:
[0010] Drosophila .alpha.-actinin gene mutants are lethal, although the flies are able to survive beyond embryogenesis with detectable muscle dysfunction present at the hatching stage (Fyrberg et al, 1998). In larval development, the mutation manifests through noticeable muscle degeneration which progressively limits mobility, and ultimately leads to death. Microscopic evaluation of mutant muscle fibers indicates that in as early as one-day old larvae, myofibrils are significantly perturbed with similar cellular pathologies to human nemaline myopathies.

Summary of Invention Paragraph:
[0018] In an additional embodiment of the present invention, there is a knockout non-human animal comprising a defective allele of a nucleic acid encoding calsarcin. In a specific embodiment, the animal further comprises two defective alleles of a nucleic acid encoding calsarcin. In an additional specific embodiment, the animal is a mouse.

Summary of Invention Paragraph:
[0019] In an additional embodiment of the present invention, there is a transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells. In specific embodiments, the promoter is constitutive, tissue specific, or inducible. In another specific embodiment the animal is a

mouse.

Summary of Invention Paragraph:
[0031] In an additional embodiment of the present invention, there is a method of inhibiting calcineurin activation of gene transcription in a cell comprising providing to said cell a fusion protein comprising calsarcin, or a calcineurin-binding fragment thereof, fused to a targeting peptide that localizes said fusion protein to a subcellular region other than a subcellular region of normal function. In a specific embodiment, a targeting peptide comprises a geranylgeranyl group, a nuclear localization signal, a myristilation signal, and an endoplasmic reticulum signal peptide. In another specific embodiment, a cell is located in an animal. In a further specific embodiment, the animal is a human. In an additional specific embodiment the method further comprises treating said animal with a compound selected from the group consisting of an ionotrope, a beta blocker, an antiarrhythmic, a diuretic, a vasodilator, a hormone antagonist, an endothelin antagonist, an angiotensin type 2 antagonist and a cytokine inhibitor/blocker.

Summary of Invention Paragraph:
[0033] In an additional embodiment of the present invention, there is a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity comprising the steps of providing a cell lacking a functional calsarcin polypeptide; contacting said cell with said candidate substance; and determining the effect of said candidate substance on said cell. In a specific embodiment, the cell is a muscle cell. In another specific embodiment, the cell has a mutation in a regulatory region of calsarcin. In a further specific embodiment the mutation is a deletion mutation, an insertion mutation, or a point mutation. In a specific embodiment, the cell has a mutation in the coding region of calsarcin. In another specific embodiment, the mutation is a deletion mutation, an insertion mutation, a frameshift mutation, a nonsense mutation, a missense mutation or a splicing mutation. In further specific embodiments, the cell is contacted in vitro or in vivo. In an additional specific embodiment, the cell is located in a non-human transgenic animal

Brief Description of Drawings Paragraph:
[0035] FIGS. 1A-1E--Predicted amino acid sequences of human and mouse calsarcin-1 and calsarcin-2. The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E).

Brief Description of Drawings Paragraph:
[0036] FIGS. 2A-D--Nucleotide sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D).

Brief Description of Drawings Paragraph:
[0037] FIG. 3--Northern blot analysis of calsarcin-1 and calsarcin-2 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-1 mRNA is predominantly detected in heart and skeletal muscle, whereas the calsarcin-2 transcript was detected in skeletal muscle of both species.

Brief Description of Drawings Paragraph:
[0043] FIG. 9--Northern blot analysis of calsarcin-3 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-3 mRNA is predominantly detected in skeletal muscle, of both species.

Detail Description Paragraph:
[0050] Current results indicate that the interaction between calsarcin-1 and

calcineurin is pertinent to the pathobiology, and ultimately to the therapy, of human heart disease. For example, familial forms of hypertrophic cardiomyopathy are caused by mutations in genes encoding proteins of the sarcomere (Seidman and Seidman, 1998) in a manner that likely involves calcineurin signaling (Marban et al, 1987). Administration of the calcineurin antagonist drugs cyclosporin A or FK-506 prevents cardiac hypertrophy in transgenic animal models of familial forms of hypertrophic cardiomyopathy (Sussman et al, 1998), but the analogous clinical trials are precluded because of toxic side effects (e.g, immunosuppression and hypertension) of existing agents.

Detail Description Paragraph:
[0051] Calcineurin antagonists also prevent cardiac hypertrophy and heart failure in some, although not all, animal models of acquired forms of cardiomyopathy that are common in human populations (Sussman et al, 1998; Ding et al, 1999; Zhang et al, 1999), but the same limitations to clinical trials apply. The relative abundance of calsarcin-1 in cardiac muscle makes it a prime target for drug development to circumvent these limitations of current calcineurin antagonists.

Detail Description Paragraph:
[0053] The significance of calcineurin-associated proteins in cardiomyopathies and muscular dystrophies is further indicated in the current invention. Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcins are likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0055] Applicants provide herein protein sequences for human calsarcin-1 (SEQ ID NO:2) and mouse calsarcin-1 (SEQ ID NO:4), human calsarcin-2 (SEQ ID NO:6), mouse calsarcin-2 (SEQ ID NO:8), human calsarcin-3 (SEQ ID NO:10) and mouse calsarcin-3 (SEQ ID NO:12). In a specific embodiment, a calcineurin associated sarcomeric protein (calsarcin) peptide, a calsarcin polypeptide or a calsarcin protein refer to calsarcin-1, calsarcin-2 or calsarcin-3. In addition to the entire calsarcin-1 molecules, the present invention also relates to fragments of the polypeptides that may or may not retain various of the functions described below. Fragments, including the N-terminus of the molecule, may be generated by genetic engineering of translation stop sites within the coding region (discussed below). Alternatively, treatment of calsarcin-1 with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, 10, and 12, of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200 or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (e.g, ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

Detail Description Paragraph:
[0072] As described in the examples, the present inventors isolated calsarcin. Given the homology between human, mouse and rat calsarcin, determined by standard means in the art, an interesting series of mutants can be created by substituting homologous regions of various proteins. This is known, in certain contexts, as "domain switching."

Detail Description Paragraph:
[0094] The present invention also provides, in another embodiment, nucleic acids encoding calsarcin. Calsarcin nucleic acids include human calsarcin-1, human calsarcin-2, human calsarcin-3, mouse calsarcin-1, mouse calsarcin-2, and mouse calsarcin-3. Nucleic acids for human calsarcin-1 (SEQ ID NO:1) and mouse calsarcin-1 (SEQ ID NO:3) have been identified. In addition, three mouse calsarcin-2 ESTs and four human calsarcin-2 ESTs were identified (see Example 1). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945. In a specific embodiment, the mouse calsarcin-2 ESTs and the human calsarcin-2 ESTs are aligned by computer programs known in the art to identify full-length mouse calsarcin-2 and human calsarcin-2 sequences respectively. The present invention is not limited in scope to these nucleic acids. However, one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in various other species (e.g, rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

Detail Description Paragraph:
[0095] In another specific embodiment, calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified. The full-length nucleic acid sequences from cDNA and genomic libraries are compared to differentiate between exon and intron sequences (Sambrook, et al, 1989). Furthermore, computer programs well known in the art use the nucleic acid sequence to generate a predicted amino acid sequence.

Detail Description Paragraph:
[0096] In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a "calsarcin nucleic acid" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the human and mouse nucleic acids disclosed herein.

Detail Description Paragraph:
[0107] Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, or 3000 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

Detail Description Paragraph:
[0117] Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or

both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Detail Description Paragraph:
[0133] In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Detail Description Paragraph:
[0139] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detail Description Paragraph:
[0154] The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grinhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Detail Description Paragraph:
[0157] Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham et al, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevac, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Detail Description Paragraph:
[0158] Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detail Description Paragraph:
[0160] Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detail Description Paragraph:
[0167] A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al, 1989).

Detail Description Paragraph:
[0213] Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Detail Description Paragraph:
[0226] To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g, incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detail Description Paragraph:
[0236] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks and drugs--in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Detail Description Paragraph:
[0237] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or

cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Detail Description Paragraph:
[0244] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Detail Description Paragraph:
[0252] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detail Description Paragraph:
[0279] Thus, in accordance with the present invention there is provided herein a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity by providing a cell lacking a functional calsarcin polypeptide, contacting the cell with a candidate substance and determining the effect of the candidate substance on the cell. The cell lacking a functional calsarcin polypeptide is described elsewhere herein and may derive from a transgenic non-human animal containing the cell, as in a cell line. The cell is preferably a muscle cell and may have a mutation in a regulatory region of calsarcin, such as a deletion, insertion or point mutation, or in the coding region, such as a deletion, insertion, frameshift, nonsense, missense or splicing mutation. The cell may be contacted in vitro or in vivo by methods well known in the art, and in a specific embodiment is located in a non-human transgenic animal.

Detail Description Paragraph:
[0294] Yeast Two-Hybrid Screens. A full-legnth mouse CnA-.alpha. cDNA, fused to the GAL4 DNA binding domain was used as bait in a two-hybrid screen of approximately 1.5.times.10.sup.6 clones of a human heart cDNA library (Clontech), as described previously (Molkentin et al, 1998). From this screen, the inventors identified a cDNA encoding calsarcin-1. Additional two-hybrid screens of the same cDNA library were performed using calsarcin-1 and calsarcin-2 as bait.

Detail Description Paragraph:
[0295] Northern blot analysis. Northern blots of RNA from human and mouse multiple tissues (Clontech) as well as from C2C12 cell extracts were performed as described (Spencer et al, 2000).

Detail Description Paragraph:
[0302] Searching public expressed sequence tag (EST) databases with the mouse calsarcin-1 cDNA sequence, human calsarcin-1 cDNA clones were identified, as well as human and mouse sequences for the related genes calsarcin-2 and calsarcin-3. A skilled artisan is aware of databases available for such searching of both protein and nucleic acid sequences, including GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html-) or commercially available databases (Celera Genomics, Inc.; Rockville, Md.; www.celera.com). Alignment of calsarcins 1-3 is demonstrated in FIG. 13.

Detail Description Paragraph:
[0303] The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E). Also provided are DNA sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D). Calsarcin-1 and -2 show the highest homology toward their amino- and carboxy-termini, whereas the intervening amino acids are less well conserved. BLATS searches with both proteins sequences did not reveal any significant homology to know proteins.

Detail Description Paragraph:
[0304] Calsarcin-2 was identified by searching the EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) with the sequence of calsarcin-1. Three mouse calsarcin-2 ESTs were identified: GenBank accession numbers (AA036142, AW742494, W29466). Additionally, four human calsarcin-2 ESTs were identified: GenBank accession numbers (AW964108, AA197193, AW000988, AA176945). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945.

Detail Description Paragraph:
[0305] Calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified.

Detail Description Paragraph:
[0313] To determine which tissues calsarcin-1 and calsarcin-2 are expressed in, Northern analysis was performed. In FIG. 3, polyA+ RNA from the indicated mouse tissues was analyzed for expression of calsarcin-1 and calsarcin-2 transcripts by methods well known in the art. The data shows a highly striated, muscle-specific expression pattern for calsarcin-1 and -2. Calsarcin-1 is specifically expressed in the heart and skeletal muscle, with two mRNAs of 1.6 and 2.6 kb in human tissues, and only a single transcript of 1.3 kb in mouse. Faint expression of calsarcin-1 was also detected in mouse lung and liver. A 1.6 kb and 1.3 kb calsarcin-2 transcript was detected exclusively in adult human and mouse skeletal muscle, respectively. The relative difference in expression level of calsarcin-1 between human and mouse skeletal muscle may reflect differences in slow-versus fast-twitch fiber composition.

Detail Description Paragraph:

[0325] N- and C-terminal truncations of calsarcin-1 were used to characterize the CnA and .alpha.-actinin interaction domains. Yeast two-hybrid assays and complementary immunoprecipitation experiments revealed that amino acids 153-200 are necessary for interaction of calsarcin with .alpha.-actinin-2 (FIG. 7). Twenty-five residues within this region are highly conserved between mouse and human calsarcin-1 and -2, suggesting that this might constitute the minimal interaction domain. Since a motif between amino acids 245-250 resembles known calcineurin dockings sites on NFAT (PxIxIT) and MCIP (PxIxxIT), the inventors tested a C-terminal truncation lacking both those residues. However, calsarcin lacking these amino acids was still able to bind can, both by two-hybrid assay (GAL4-calsarcin 85-240) and coimmunoprecipitation (Myc-calsarcin 1-240). In contrast, a calsarcin-1 mutant lacking residues 217-264 was unable to bind CnA, implying that residues 217-240 are necessary for binding. Mapping of the interaction domain on CnA revealed that the calsarcin-interacting domain residues within the catalytic region, whereas the calsarcin-1 interacting domain of .alpha.-actinin maps to the second and third spectrin-like repeats.

Detail Description Paragraph:
[0327] Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcin-1 is likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0346] Barski O A, Gabbay K H, Bohren K M. Characterization of the human aldehyde reductase gene and promoter. Genomics 60(2):188-98 (1999).

Detail Description Paragraph:
[0347] Beggs A H, Byers T J, Knoll J H, Boyce F M, Bruns G A, Kunkel L M. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J Biol. Chem. May 5;267(13):9281-8 (1992).

Detail Description Paragraph:
[0349] Bhavsar P K, Brand N J, Yacoub N H, Barton P J R. Isolation and characterization of the human cardiac troponin I gene (TNNI3). Genomics. Jul 1;35 (1):11-23 (1996).

Detail Description Paragraph:
[0376] Landon F, Gache Y, Touitou H, Olomucki A. Properties of two isoforms of human blood platelet alpha-actinin. Eur J Biochem. Dec 2;153(2):231-7 (1985).

Detail Description Paragraph:
[0377] LaPointe M C, Wu G, Garami M, Yang X P, Gardner D G. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes. Hypertension. March;27(3 Pt 2):715-22 (1996).

Detail Description Paragraph:
[0397] Ritchie M E. Characterization of human B creatine kinase gene regulation in the heart in vitro and in vivo. J Biol. Chem. October 11;271(41):25485-91 (1996).

Detail Description Table CWU:
2TABLE 2 Promoter and/or Enhancer Promoter/Enhancer References Immunoglobulin Heavy Chain Banerji et al., 1983; Gilles et al., 1983; Grosschedl et al., 1985; Atchinson et al., 1986, 1987; Imler et al., 1987; Weinberger et al., 1984; Kiledjian et al.,

1988; Porton et al.; 1990 Immunoglobulin Light Chain Queen et al., 1983; Picard et al., 1984 T-Cell Receptor Luria et al., 1987; Winoto et al., 1989; Redondo et al.; 1990 HLA DQ a and/or DQ Sullivan et al., 1987 -Interferon Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988 Interleukin-2 Greene et al., 1989 Interleukin-2 Receptor Greene et al., 1989; Lin et al., 1990 MHC Class II 5 Koch et al., 1989 MHC Class II HLA-DRa Sherman et al., 1989 -Actin Kawamoto et al., 1988; Ng et al.; 1989 Muscle Creatine Kinase (MCK) Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., 1989 Prealbumin (Transthyretin) Costa et al., 1988 Elastase I Ornitz et al., 1987 Metallothionein (MTII) Kerin et al., 1987; Culotta et al., 1989 Collagenase Pinkert et al., 1987; Angel et al., 1987a Albumin Pinkert et al., 1987; Tronche et al., 1989, 1990 -Fetoprotein Godbout et al., 1988; Campere et al., 1989 t-Globin Bodine et al., 1987; Perez-Stable et al., 1990 Globin Trudel et al., 1987 c-fos Cohen et al., 1987 c-HA-ras Triesman, 1986; Deschamps et al., 1985 Insulin Edlund et al., 1985 Neural Cell Adhesion Molecule Hirsh et al., 1990 (NCAM) .sub.1-Antitrypain Latimer et al., 1990 H2B (TH2B) Histone Hwang et al., 1990 Mouse and/or Type I Collagen Ripe et al., 1989 Glucose-Regulated Proteins Chang et al., 1989 (GRP94 and GRP78) Rat Growth Hormone Larsen et al., 1986 Human Serum Amyloid A (SAA) Edbrooke et al., 1989 Troponin I (TN I) Yutzey et al., 1989 Platelet-Derived Growth Factor Pech et al., 1989 (PDGF) Duchenne Muscular Dystrophy Klamut et al., 1990 SV40 Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Firak et al., 1986; Herr et al., 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988 Polyoma Swartzendruber et al., 1975; Vasseur et al., 1980; Katinka et al., 1980, 1981; Tyndell et al., 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and/or Villarreal, 1988 Retroviruses Kriegler et al., 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander et al., 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman et al., 1989 Papilloma Virus Campo et al., 1983; Lusky et al., 1983; Spandidos and/or Wilkie, 1983; Spalholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens et al., 1987; Glue et al., 1988 Hepatitis B Virus Bulla et al., 1986; Jameel et al., 1986; Shaul et al., 1987; Spandau et al., 1988; Vannice et al., 1988 Human Immunodeficiency Virus Muesing et al., 1987; Hauber et al., 1988; Jakobovits et al., 1988; Feng et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp et al., 1989; Braddock et al., 1989 Cytomegalovirus (CMV) Weber et al., 1984; Boshart et al., 1985; Foecking et al., 1986 Gibbon Ape Leukemia Virus Holbrook et al., 1987; Quinn et al., 1989

CLAIMS:

22. A knockout non-human animal comprising a defective allele of a nucleic acid encoding a calcineurin associated sarcomeric protein (calsarcin).

25. A transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells.

93. The method of claim 92 wherein said animal is a human.

105. The method of claim 105, wherein said cell is located in a non-human transgenic animal.

☐ 5. Document ID: US 20030078376 A1

L4: Entry 5 of 10 File: PGPB Apr 24, 2003

DOCUMENT-IDENTIFIER: US 20030078376 A1
TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

Summary of Invention Paragraph:
[0006] Recent studies of calcineurin signaling in striated myocytes of heart and skeletal muscle have expanded the scope of important physiological and pathological events controlled by this ubiquitously expressed protein. Forced expression of a constitutively active form of calcineurin in hearts of transgenic mice promotes cardiac hypertrophy that progresses to dilated cardiomyopathy, heart failure, and death, in a manner that recapitulates features of human disease (Molkentin et al., 1998, herein incorporated by reference). Moreover, hypertrophy and heart failure in these animals, and in certain other animal models of cardiomyopathy, are prevented by administration of the calcineurin antagonist drugs cyclosporin A or FK-506 (Sussman et al., 1998). In skeletal muscles, calcineurin signaling is implicated both in hypertrophic growth stimulated by insulin-like growth factor-1 (Musaro et al., 1999; Semsarian et al., 1999), and in the control of specialized programs of gene expression that establish distinctive myofiber subtypes (Chin et al., 1998; Dunn et al., 1999). These observations have stimulated interest in the therapeutic potential of modifying calcineurin activity selectively in muscle cells while avoiding unwanted consequences of altered calcineurin signaling in other cell types (Sigal et al., 1991).

Summary of Invention Paragraph:
[0010] Drosophila .alpha.-actinin gene mutants are lethal, although the flies are able to survive beyond embryogenesis with detectable muscle dysfunction present at the hatching stage (Fyrberg et al., 1998). In larval development, the mutation manifests through noticeable muscle degeneration which progressively limits mobility, and ultimately leads to death. Microscopic evaluation of mutant muscle fibers indicates that in as early as one-day old larvae, myofibrils are significantly perturbed with similar cellular pathologies to human nemaline myopathies.

Summary of Invention Paragraph:
[0018] In an additional embodiment of the present invention, there is a knockout non-human animal comprising a defective allele of a nucleic acid encoding calsarcin. In a specific embodiment, the animal further comprises two defective alleles of a nucleic acid encoding calsarcin. In an additional specific embodiment, the animal is a mouse.

Summary of Invention Paragraph:
[0019] In an additional embodiment of the present invention, there is a transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells. In specific embodiments, the promoter is constitutive, tissue specific, or inducible. In another specific embodiment the animal is a mouse.

Summary of Invention Paragraph:
[0031] In an additional embodiment of the present invention, there is a method of inhibiting calcineurin activation of gene transcription in a cell comprising providing to said cell a fusion protein comprising calsarcin, or a calcineurin-binding fragment thereof, fused to a targeting peptide that localizes said fusion protein to a subcellular region other than a subcellular region of normal function. In a specific embodiment, a targeting peptide comprises a geranylgeranyl group, a

nuclear localization signal, a myristilation signal, and an endoplasmic reticulum signal peptide. In another specific embodiment, a cell is located in an animal. In a further specific embodiment, the animal is a human. In an additional specific embodiment the method further comprises treating said animal with a compound selected from the group consisting of an ionotrope, a beta blocker, an antiarrhythmic, a diuretic, a vasodilator, a hormone antagonist, an endothelin antagonist, an angiotensin type 2 antagonist and a cytokine inhibitor/blocker.

Summary of Invention Paragraph:
[0033] In an additional embodiment of the present invention, there is a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity comprising the steps of providing a cell lacking a functional calsarcin polypeptide; contacting said cell with said candidate substance; and determining the effect of said candidate substance on said cell. In a specific embodiment, the cell is a muscle cell. In another specific embodiment, the cell has a mutation in a regulatory region of calsarcin. In a further specific embodiment the mutation is a deletion mutation, an insertion mutation, or a point mutation. In a specific embodiment, the cell has a mutation in the coding region of calsarcin. In another specific embodiment, the mutation is a deletion mutation, an insertion mutation, a frameshift mutation, a nonsense mutation, a missense mutation or a splicing mutation. In further specific embodiments, the cell is contacted in vitro or in vivo. In an additional specific embodiment, the cell is located in a non-human transgenic animal

Brief Description of Drawings Paragraph:
[0035] FIGS. 1A-1E--Predicted amino acid sequences of human and mouse calsarcin-1 and calsarcin-2. The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E).

Brief Description of Drawings Paragraph:
[0036] FIGS. 2A-D--Nucleotide sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D).

Brief Description of Drawings Paragraph:
[0037] FIG. 3--Northern blot analysis of calsarcin-1 and calsarcin-2 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-1 mRNA is predominantly detected in heart and skeletal muscle, whereas the calsarcin-2 transcript was detected in skeletal muscle of both species.

Brief Description of Drawings Paragraph:
[0043] FIG. 9--Northern blot analysis of calsarcin-3 in adult human and mouse tissues. Calsarcin transcripts were detected by Northern analysis of the indicated human and mouse tissues. Calsarcin-3 mRNA is predominantly detected in skeletal muscle, of both species.

Detail Description Paragraph:
[0050] Current results indicate that the interaction between calsarcin-1 and calcineurin is pertinent to the pathobiology, and ultimately to the therapy, of human heart disease. For example, familial forms of hypertrophic cardiomyopathy are caused by mutations in genes encoding proteins of the sarcomere (Seidman and Seidman, 1998) in a manner that likely involves calcineurin signaling (Marban et al., 1987). Administration of the calcineurin antagonist drugs cyclosporin A or FK-506 prevents cardiac hypertrophy in transgenic animal models of familial forms of hypertrophic cardiomyopathy (Sussman et al., 1998), but the analogous clinical trials are precluded because of toxic side effects (e.g., immunosuppression and hypertension) of existing agents.

Detail Description Paragraph:
[0051] Calcineurin antagonists also prevent cardiac hypertrophy and heart failure in some, although not all, animal models of acquired forms of cardiomyopathy that are common in human populations (Sussman et al., 1998; Ding et al., 1999; Zhang et al., 1999), but the same limitations to clinical trials apply. The relative abundance of calsarcin-1 in cardiac muscle makes it a prime target for drug development to circumvent these limitations of current calcineurin antagonists.

Detail Description Paragraph:
[0053] The significance of calcineurin-associated proteins in cardiomyopathies and muscular dystrophies is further indicated in the current invention. Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcins are likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0055] Applicants provide herein protein sequences for human calsarcin-1 (SEQ ID NO:2) and mouse calsarcin-1 (SEQ ID NO:4), human calsarcin-2 (SEQ ID NO:6), mouse calsarcin-2 (SEQ ID NO:8), human calsarcin-3 (SEQ ID NO:10) and mouse calsarcin-3 (SEQ ID NO:12). In a specific embodiment, a calcineurin associated sarcomeric protein (calsarcin) peptide, a calsarcin polypeptide or a calsarcin protein refer to calsarcin-1, calsarcin-2 or calsarcin-3. In addition to the entire calsarcin-1 molecules, the present invention also relates to fragments of the polypeptides that may or may not retain various of the functions described below. Fragments, including the N-terminus of the molecule, may be generated by genetic engineering of translation stop sites within the coding region (discussed below). Alternatively, treatment of calsarcin-1 with proteolytic enzymes, known as proteases, can produce a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, 10, and 12, of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200 or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (e.g., ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

Detail Description Paragraph:
[0072] As described in the examples, the present inventors isolated calsarcin. Given the homology between human, mouse and rat calsarcin, determined by standard means in the art, an interesting series of mutants can be created by substituting homologous regions of various proteins. This is known, in certain contexts, as "domain switching."

Detail Description Paragraph:
[0094] The present invention also provides, in another embodiment, nucleic acids encoding calsarcin. Calsarcin nucleic acids include human calsarcin-1, human calsarcin-2, human calsarcin-3, mouse calsarcin-1, mouse calsarcin-2, and mouse calsarcin-3. Nucleic acids for human calsarcin-1 (SEQ ID NO:1) and mouse calsarcin-1 (SEQ ID NO:3) have been identified. In addition, three mouse calsarcin-2 ESTs and four human calsarcin-2 ESTs were identified (see Example 1). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank

No. AA197193; GenBank No. AW000988; and GenBank No. AA176945. In a specific embodiment, the mouse calsarcin-2 ESTs and the human calsarcin-2 ESTs are aligned by computer programs known in the art to identify full-length mouse calsarcin-2 and human calsarcin-2 sequences respectively. The present invention is not limited in scope to these nucleic acids. However, one of ordinary skill in the art could, using these nucleic acids, readily identify related homologs in various other species (e.g., rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

Detail Description Paragraph:
[0095] In another specific embodiment, calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified. The full-length nucleic acid sequences from cDNA and genomic libraries are compared to differentiate between exon and intron sequences (Sambrook, et al., 1989). Furthermore, computer programs well known in the art use the nucleic acid sequence to generate a predicted amino acid sequence.

Detail Description Paragraph:
[0096] In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, a "calsarcin nucleic acid" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the human and mouse nucleic acids disclosed herein.

Detail Description Paragraph:
[0107] Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, or 3000 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

Detail Description Paragraph:
[0117] Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Detail Description Paragraph:
[0133] In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to

achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Detail Description Paragraph:
[0140] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detail Description Paragraph:
[0155] The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Detail Description Paragraph:
[0158] Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevac, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Detail Description Paragraph:
[0159] Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detail Description Paragraph:
[0161] Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about

which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detail Description Paragraph:
[0168] A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

Detail Description Paragraph:
[0214] Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Detail Description Paragraph:
[0227] To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detail Description Paragraph:
[0240] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks and drugs--in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Detail Description Paragraph:
[0241] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Detail Description Paragraph:
[0249] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral

administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Detail Description Paragraph:
[0257] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detail Description Paragraph:
[0282] Thus, in accordance with the present invention there is provided herein a method of screening for a candidate substance for anti-cardiomyopic hypertrophy activity or anti-heart failure activity by providing a cell lacking a functional calsarcin polypeptide, contacting the cell with a candidate substance and determining the effect of the candidate substance on the cell. The cell lacking a functional calsarcin polypeptide is described elsewhere herein and may derive from a transgenic non-human animal containing the cell, as in a cell line. The cell is preferably a muscle cell and may have a mutation in a regulatory region of calsarcin, such as a deletion, insertion or point mutation, or in the coding region, such as a deletion, insertion, frameshift, nonsense, missense or splicing mutation. The cell may be contacted in vitro or in vivo by methods well known in the art, and in a specific embodiment is located in a non-human transgenic animal.

Detail Description Paragraph:
[0297] Yeast Two-Hybrid Screens. A full-legnth mouse CnA-.alpha. cDNA, fused to the GAL4 DNA binding domain was used as bait in a two-hybrid screen of approximately 1.5.times.10.sup.6 clones of a human heart cDNA library (Clontech), as described previously (Molkentin et al., 1998). From this screen, the inventors identified a cDNA encoding calsarcin-1. Additional two-hybrid screens of the same cDNA library were performed using calsarcin-1 and calsarcin-2 as bait.

Detail Description Paragraph:
[0298] Northern blot analysis. Northern blots of RNA from human and mouse multiple tissues (Clontech) as well as from C2C12 cell extracts were performed as described (Spencer et al., 2000).

Detail Description Paragraph:
[0305] Searching public expressed sequence tag (EST) databases with the mouse calsarcin-1 cDNA sequence, human calsarcin-1 cDNA clones were identified, as well

as human and mouse sequences for the related genes calsarcin-2 and calsarcin-3. A skilled artisan is aware of databases available for such searching of both protein and nucleic acid sequences, including GenBank (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html-) or commercially available databases (Celera Genomics, Inc.; Rockville, Md.; www.celera.com). Alignment of calsarcins 1-3 is demonstrated in FIG. 13.

Detail Description Paragraph:
[0306] The deduced amino acid sequences of human calsarcin-1 (FIG. 1A), mouse calsarcin-1 (FIG. 1B), human calsarcin-2 (FIG. 1C) and mouse calsarcin-2 (FIG. 1D) are shown, along with an amino acid alignment of the mouse proteins (FIG. 1E). Also provided are DNA sequences for human calsarcin-1 (FIG. 2A), mouse calsarcin-1 (FIG. 2B), human calsarcin-2 (FIG. 2C) and mouse calsarcin-2 (FIG. 2D). Calsarcin-1 and -2 show the highest homology toward their amino- and carboxy-termini, whereas the intervening amino acids are less well conserved. BLATS searches with both proteins sequences did not reveal any significant homology to know proteins.

Detail Description Paragraph:
[0307] Calsarcin-2 was identified by searching the EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) with the sequence of calsarcin-1. Three mouse calsarcin-2 ESTs were identified: GenBank accession numbers (AA036142, AW742494, W29466). Additionally, four human calsarcin-2 ESTs were identified: GenBank accession numbers (AW964108, AA197193, AW000988, AA176945). The mouse calsarcin-2 ESTs are as follows: GenBank No. AA036142; GenBank No. AW742494; and GenBank No. W29466. The human calsarcin-2 ESTs are as follows: GenBank No. AW964108; GenBank No. AA197193; GenBank No. AW000988; and GenBank No. AA176945.

Detail Description Paragraph:
[0308] Calsarcin-3 was discovered "in silico" by comparing calsarcin 1 and calsarcin 2 sequences with the database. Human genomic DNA (AC 008453.3; public not Celera database) containing several homologous sequences was confirmed to be exons of calsarcin-3. Primers were designed and a human skeletal muscle library was screened for the full-length cDNA for human calsarcin-3 (FIG. 5). Similarly, a mouse skeletal library was screened and several independent and overlapping clones encoding for mouse calscarcin-3 were identified.

Detail Description Paragraph:
[0316] To determine which tissues calsarcin-1 and calsarcin-2 are expressed in, Northern analysis was performed. In FIG. 3, polyA+ RNA from the indicated mouse tissues was analyzed for expression of calsarcin-1 and calsarcin-2 transcripts by methods well known in the art. The data shows a highly striated, muscle-specific expression pattern for calsarcin-1 and -2. Calsarcin-1 is specifically expressed in the heart and skeletal muscle, with two mRNAs of 1.6 and 2.6 kb in human tissues, and only a single transcript of 1.3 kb in mouse. Faint expression of calsarcin-1 was also detected in mouse lung and liver. A 1.6 kb and 1.3 kb calsarcin-2 transcript was detected exclusively in adult human and mouse skeletal muscle, respectively. The relative difference in expression level of calsarcin-1 between human and mouse skeletal muscle may reflect differences in slow- versus fast-twitch fiber composition.

Detail Description Paragraph:
[0328] N- and C-terminal truncations of calsarcin-1 were used to characterize the CnA and .alpha.-actinin interaction domains. Yeast two-hybrid assays and complementary immunoprecipitation experiments revealed that amino acids 153-200 are necessary for interaction of calsarcin with .alpha.-actinin-2 (FIG. 7). Twenty-five residues within this region are highly conserved between mouse and human calsarcin-1 and -2, suggesting that this might constitute the minimal interaction domain. Since a motif between amino acids 245-250 resembles known calcineurin dockings sites on NFAT (PxIxIT) and MCIP (PxIxxIT), the inventors tested a C-terminal truncation lacking both those residues. However, calsarcin lacking these amino

acids was still able to bind can, both by two-hybrid assay (GAL4-calsarcin 85-240) and coimmunoprecipitation (Myc-calsarcin 1-240). In contrast, a calsarcin-1 mutant lacking residues 217-264 was unable to bind CnA, implying that residues 217-240 are necessary for binding. Mapping of the interaction domain on CnA revealed that the calsarcin-interacting domain residues within the catalytic region, whereas the calsarcin-1 interacting domain of .alpha.--actinin maps to the second and third spectrin-like repeats.

Detail Description Paragraph:
[0330] Based on their interactions and colocalization in vivo, it also is proposed herein that calsarcin-1 links calcineurin to the Z-band where it can sense changes in calcium signaling in the myocyte and potentially transduce a hypertrophic signal (FIG. 8). Calsarcin-1, and/or other calsarcin proteins, such as calsarcin-2 or calsarcin-3, may also play structural and/or mechanosensory roles in cardiac and skeletal myocytes through modulation of the Z-band and its association with other proteins in the cell. The Z-band has been shown to play important roles in regulating muscle cell structure and function. Thus, calsarcin-1 is likely to be intimately involved in these processes and is a strong candidate for a gene involved in human cardiomyopathies and muscular dystrophies.

Detail Description Paragraph:
[0349] Barski O A, Gabbay K H, Bohren K M. Characterization of the human aldehyde reductase gene and promoter. Genomics 60(2):188-98 (1999).

Detail Description Paragraph:
[0350] Beggs A H, Byers T J, Knoll J H, Boyce F M, Bruns G A, Kunkel L M. Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. J Biol Chem. May 5;267(13):9281-8 (1992).

Detail Description Paragraph:
[0352] Bhavsar P K, Brand N J, Yacoub N H, Barton P J R. Isolation and characterization of the human cardiac troponin I gene (TNNI3).Genomics. Jul 1;35 (1):11-23 (1996).

Detail Description Paragraph:
[0379] Landon F, Gache Y, Touitou H, Olomucki A. Properties of two isoforms of human blood platelet alpha-actinin. Eur J Biochem. Dec 2;153(2):23 1-7 (1985).

Detail Description Paragraph:
[0380] LaPointe M C, Wu G, Garami M, Yang X P, Gardner D G. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes. Hypertension. Mar;27(3 Pt 2):715-22 (1996).

Detail Description Paragraph:
[0400] Ritchie M E. Characterization of human B creatine kinase gene regulation in the heart in vitro and in vivo. J Biol Chem. Oct 11;271(41):25485-91 (1996).

Detail Description Table CWU:
2TABLE 2 Promoter and/or Enhancer Promoter/ Enhancer References Immuno- Banerji et al., 1983; Gilles et al., 1983; Grosschedl globulin et al., 1985; Atchinson et al., 1986, 1987; Imler et al., Heavy Chain 1987; Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al.; 1990 Immunoglobulin Queen et al., 1983; Picard et al., 1984 Light Chain T-Cell Receptor Luria et al., 1987; Winoto et al., 1989; Redondo et at.; 1990 HLA DQ a Sullivan et al., 1987 and/or DQ -Interferon Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988 Interleukin-2 Greene et al., 1989 Interleukin-2 Greene et al., 1989; Lin et al., 1990 Receptor MHC Class II 5 Koch et al., 1989 MHC Class II Sherman et al., 1989 HLA-DRa -Actin Kawamoto et al., 1988; Ng et al.; 1989 Muscle Creatine Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., Kinase (MCK) 1989 Prealbumin Costa et al., 1988 (Transthyretin) Elastase I Ornitz et al., 1987 Metallothionein Karin et al., 1987; Culotta et al., 1989 (MTII)

Collagenase Pinkert et al., 1987; Angel et al., 1987a Albumin Pinkert et al., 1987; Tronche et al., 1989, 1990 -Fetoprotein Godbout et al., 1988; Campere et al., 1989 t-Globin Bodine et al., 1987; Perez-Stable et al., 1990 -Globin Trudel et al., 1987 c-fos Cohen et al., 1987 c-HA-ras Triesman, 1986; Deschamps et al., 1985 Insulin Edlund et al., 1985 Neural Cell Hirsh et al., 1990 Adhesion Molecule (NCAM) .sub.]- Antitrypain Latimer et al., 1990 H2B (TH2B) Hwang et al., 1990 Histone Mouse and/or pe et al., 1989 Type I Collagen Glucose- Chang et al., 1989 Regulated Proteins (GRP94 and GRP78) Rat Growth Larsen et al., 1986 Hormone Human Serum dbrooke et al., 1989 Amyloid A (SAA) Troponin I (TN I) Yutzey et al., 1989 Platelet-Derived Pech et al., 1989 Growth Factor (PDGF) Duchenne Klamut et al., 1990 Muscular Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., Dystrophy SV40 1985; Firak et al., 1986; Herr et al, 1986; Imbra et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Ondek et al., 1987; Kuhl et al., 1987; Schaffner et al., 1988 Polyoma Swartzendruber et al., 1975; Vasseur et al., 1980; Katink et al., 1980, 1981; Tyndell et al., 1981; Dandolo et al., 1983; de Villiers et al., 1984; Hen et al., 1986; Satake et al., 1988; Campbell and/or Villarreal, 1988 Retroviruses Kriegler et al., 1982, 1983; Levinson et al., 1982; Kriegler et al., 1983, 1984a, b, 1988; Bosze et al., 1986; Miksicek et al., 1986; Celander et al., 1987; Thiesen et al., 1988; Celander et al., 1988; Choi et al., 1988; Reisman et al., 1989 Papilloma Campo et al., 1983; Lusky et al., 1983; Spandidos Virus and/or Wilkie, 1983; Spalholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Gloss et al., 1987; Hirochika et al., 1987; Stephens et al., 1987; Glue et al., 1988 Hepatitis B Bulla et al., 1986; Jameel et al., 1986; Shaul et al., Virus 1987; Spandau et al., 1988, Vannice et al., 1988 Human Immuno- Muesing et al., 1987; Hauber et al., 1988; Jakobovits deficiency Virus et al., 1988; Feng et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkhout et al., 1989; Laspia et al., 1989; Sharp et al., 1989; Braddock et al., 1989 Cytomegalovirus Weber et al. , 1984; Boshart et al., 1985, (CMV) Foecking et al., 1986 Gibbon Ape Holbrook et al., 1987; Quinn et al., 1989 Leukemia Virus

CLAIMS:

22. A knockout non-human animal comprising a defective allele of a nucleic acid encoding a calcineurin associated sarcomeric protein (calsarcin).

25. A transgenic non-human animal comprising an expression cassette, wherein said cassette comprises a nucleic acid encoding a calsarcin polypeptide under the control of a promoter active in eukaryotic cells.

93. The method of claim 92 wherein said animal is a human.

105. The method of claim 105, wherein said cell is located in a non-human transgenic animal.

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L4: Entry 6 of 10 File: PGPB Oct 17, 2002

DOCUMENT-IDENTIFIER: US 20020150953 A1
TITLE: Methods and compositions relating to muscle selective calcineurin interacting protein (MCIP)

Summary of Invention Paragraph:

[0006] Recent studies of calcineurin signaling in striated myocytes of heart and skeletal muscle have expanded the scope of important physiological and pathological events controlled by this ubiquitously expressed protein. Forced expression of a constitutively active form of calcineurin in hearts of transgenic mice promotes cardiac hypertrophy that progresses to dilated cardiomyopathy, heart failure, and death, in a manner that recapitulates features of human disease (Molkentin et al., 1998). Moreover, hypertrophy and heart failure in these animals, and in certain other animal models of cardiomyopathy, are prevented by administration of the calcineurin antagonist drugs cyclosporin A or FK-506 (Sussman et al., 1998). In skeletal muscles, calcineurin signaling is implicated both in hypertrophic growth stimulated by insulin-like growth factor-1 (Musaro et al., 1999; Semsarian et al., 1999), and in the control of specialized programs of gene expression that establish distinctive myofiber subtypes (Chin et al., 1998; Dunn et al., 1999). These observations have stimulated interest in the therapeutic potential of modifying calcineurin activity selectively in muscle cells while avoiding unwanted consequences of altered calcineurin signaling in other cell types (Sigal et al., 1991).

Brief Description of Drawings Paragraph:
[0030] FIG. 3--Splice variants of MCIP1 and MCIP2 inhibit calcineurin signaling. C2C12 cells were cotransfected with the Mb-luc reporter plasmid, an empty control vector (pCI), or expression vectors encoding constitutively active calcineurin (CnA*), human MCIP1 (hMCIP1), two splice variants of murine MCIP1 (mMCIP1/exon 1 or mMCIP1/exon4), or murine MCIP2 (mMCIP2) as indicated. Cells were harvested 48 h after transfection. Luciferase expression was determined in the absence (open bar) or presence (filled bars) of constitutively active calcineurin (CnA*). Data were calculated as described in FIGS. 1A-B. All results are corrected for variations in transfection efficiency by normalization to expression of a co-transfected pCMV-lacZ plasmid. FIGS. 4A-B--MCIP1 binds the catalytic domain of calcineurin A. FIG. 4A: schematic depiction of functional domains of calcineurin A, as defined by previous studies (1), and including the catalytic domain, binding sites for calcineurin B (B) and calmodulin (M), and the carboxyl-terminal autoinhibitory domain (I). Truncated forms of calcineurin A are identified by their termination at specific amino acid (aa) residues corresponding to positions within the full-length protein, and by their binding to MCIP1. FIG. 4B: calcineurin A proteins were translated in rabbit reticulocyte lysates and labeled with [.sup.35S]methionine. Recombinant GST-MCIP1 was purified from bacteria and coupled to glutathione-agarose beads. Binding of truncated forms of calcineurin A to GST-MCIP1 was compared with GST alone, and to 25% of the total pool of metabolically labeled protein included in the binding reaction (Input). Luciferase failed to interact with GST-MCIP1, serving as a negative control (data not shown). Proteins were separated by SDS-PAGE and visualized by autoradiography. FIGS. 5A-B--Conserved regions of MCIP1 mediate the interaction with calcineurin A. FIG. 5A: schematic depiction of MCIP1 illustrating amino acid sequence motifs conserved with yeast Rex1p (24) and mammalian NFAT (3) proteins, and including an SP repeat region and calcineurin docking motif (P). Truncated forms of MCIP1 are identified by their termination at specific amino acid (aa) residues corresponding to positions within the full-length protein, and by their binding to calcineurin A. FIG. 5B: calcineurin A (amino acids 1-398) was translated in rabbit reticulocyte lysates and labeled with [.sup.35S] methionine. Recombinant full-length or truncated forms of GST-MCIP1 were purified from bacteria and coupled to glutathione-agarose beads. Binding of calcineurin A to each variant of GST-MCIP1 was compared with GST alone, and to 25% of the total pool of metabolically labeled protein included in the binding reaction (input). Proteins were separated by SDS-PAGE and visualized by autoradiography.

Brief Description of Drawings Paragraph:
[0033] FIGS. 8A-C--An intragenic calcineurin response element from the MCIP1 gene. FIG. 8A. Schematic representation of the organization of the human MCIP1 (DSCR1) gene, indicating four alternative initial exons (E1-E4) and three exons common to all forms of MCIP1 mRNA (E5-E7)34. The nucleotide sequence flanking Exon 4 is shown

to illustrate the presence of 15 consensus binding sites for NF-AT transcription factors (boxes). The first nucleotide of exon 4 is designated as +1. FIG. 8B. MCIP1 -luciferase reporter plasmids. Plasmids were constructed to link defined genomic segments proximal to exon 4 of the human MCIP1 gene to a luciferase reporter gene. The numbers of NF-AT consensus binding sites contained within each segment are shown in parenthesis. FIG. 8D. Transient transfection assays of MCIP1 -luciferase reporter plasmids. Results were corrected for variations in transfection efficiency by normalization to expression of a co-transfected pCMV-lacZ plasmid. Fold activation was determined relative to the basal activity of the *874 to +30 MCIP1 -luciferase reporter construct. Histograms represent mean values + SEM of 3 independent transfections of C2C12 myogenic cells.

Brief Description of Drawings Paragraph:
[0035] FIG. 10--Design and expression of the .alpha.-MHC-hMCIP1 transgene. Schematic illustration of components of the transgene, including a 5.5 kb .alpha.-MHC promoter fragment with three non-coding exons (E1, E2, E3) and intervening non-transcribed segments of the .alpha.-MHC gene, followed by a full length human MCIP1 cDNA with a carboxyl terminal epitope tag (HA) and a polyadenylation (pA) signal from the human growth hormone gene. The lower line illustrates the unexpected pattern of mRNA splicing observed in vivo, resulting in translation of a truncated protein (DhMICP1) initiated at amino acid 81 relative to the wild-type (WT) protein.

Detail Description Paragraph:
[0041] As discussed above, Rex1p (YKL159c) is a calcineurin-binding protein of Saccharomyces cerevisiae . A preliminary report noted that this small 24 kDa protein inhibited calcineurin signaling when overexpressed in yeast (Kingsbury and Cunningham 1998). A 30-amino acid segment of Rex1p shares homology to two different genes identified in the human gene sequence data base, DSCR1 and ZAKI-4. DSCR1 was so designated because it resides within the "Down syndrome critical region" of human chromosome 21 (Fuentes et al., 1997). Individuals trisomic for this region, which is estimated to encode 50-100 different proteins, display features of the Down syndrome phenotype. ZAKI-4 was identified from a human fibroblast cell line in a screen for genes that are transcriptionally activated in response to thyroid hormone (Miyazaki et al., 1996).

Detail Description Paragraph:
[0047] Recently, additions to the human genome data base includes a third MCIP gene, with two splice variants. This gene is located on chromosome 1, with the variants having accession numbers AF176116 (SEQ ID NOS:16 and 17) and AF176117 (SEQ ID NOS:5 and 6). These gene products are termed MCIP3a and MCIP3b by the inventors. There also appears to be a splice variant of MCIP2, now called MCIP2a and MCIP2b.

Detail Description Paragraph:
[0049] Current results indicate that the interaction between MCIP and calcineurin is pertinent to the pathobiology, and ultimately to the therapy, of human disease, For example, familial forms of hypertrophic cardiomyopathy are caused by mutations in genes encoding proteins of the sarcomere (Seidman and Seidman, 1998), in a manner that is likely to involve calcineurin signaling (Marban et al., 1987). Administration of the calcineurin antagonist drugs cyclosporin A or FK-506 prevents cardiac hypertrophy in transgenic animal models of familial forms of hypertrophic cardiomyopathy (Sussman et al., 1998), but the analogous clinical trials are precluded because of toxic side effects (e.g., immunosuppression and hypertension) of existing agents.

Detail Description Paragraph:
[0051] Calcineurin antagonists also prevent cardiac hypertrophy and heart failure in some, although not all, animal models of acquired forms of cardiomyopathy that are common in human populations (Sussman et al., 1998; Ding et al., 1999; Zhang et al., 1999), but the same limitations to clinical trials apply. The relative

abundance of MCIP1 in cardiac muscle recommends it as a target for drug development to circumvent these limitations of current calcineurin antagonists.

Detail Description Paragraph:
[0053] The human gene (DSCR1) encoding MCIP1 is one of 50-100 genes that reside within a critical region of chromosome 21 (Fuentes et al., 1997; Fuentes et al., 1995), trisomy of which gives rise to the complex developmental abnormalities of Down syndrome, which include cardiac abnormalities and skeletal muscle hypotonia as prominent features (Epstein, 1995). ZAKI-4 was identified from a human fibroblast cell line in a screen for genes that are transcriptionally activated in response to thyroid hormone (Miyazaki et al, 1996).

Detail Description Paragraph:
[0054] Applicants provide protein sequences for human MCIP1, MCIP2 and MCIP3 (SEQ ID NOS:2, 4, 6 and 17) and mouse MCIP1 and MCIP2 (SEQ ID NOS:8 and 10). In addition to the entire MCIP1, MCIP2 and MCIP3 molecules, the present invention also relates to fragments of the polypeptides that may or may not retain various of the functions described below. Fragments, including the N-terminus of the molecule may be generated by genetic engineering of translation stop sites within the coding region (discussed below). Alternatively, treatment of the MCIPs with proteolytic enzymes, known as proteases, can produces a variety of N-terminal, C-terminal and internal fragments. Examples of fragments may include contiguous residues of SEQ ID NOS:2, 4, 6, 8, 10 and 17 of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, 200, 300, 400 or more amino acids in length. These fragments may be purified according to known methods, such as precipitation (e.g., ammonium sulfate), HPLC, ion exchange chromatography, affinity chromatography (including immunoaffinity chromatography) or various size separations (sedimentation, gel electrophoresis, gel filtration).

Detail Description Paragraph:
[0056] MCIP1 is the product of a human gene previously called,DSCR1, and it includes several domains of proven or potential biological interest: an acidic domain (EKEEEEEME), a serine-proline motif (SPPASPP), a leucine-rich putative DNA binding domain (LHKTEFLGKEMKLYFAQTL), and regions with the characteristics of an SH3 or SH2 domain ligand (HLAPPNPDK and PEYTPI, respectively). Fuentes et al. (1997). Multiple versions of MCIP1 exist due to four alternative first exons, which are alternatively joined to Exons 5-7. It is the form of MCIP1 initiated at Exon 4 that is transcriptionally induced by calcineurin activity. MCIP2, also known as ZAKI-4, is a 192 AA polypeptide having 62% homology with MCIP 1. Proline and valine residues are found with abundance within MCIP2. Miyazaki et al. (1996).

Detail Description Paragraph:
[0093] The present invention also provides, in another embodiment, genes encoding MCIP1 and MCIP2. Genes for human MCIP1 (SEQ ID NO:1), MCIP2 (SEQ ID NO:3) and MCIP3 (SEQ ID:5 and 16) have been identified. Also provided are MCIP1 and MCIP2 from mouse (SEQ ID NOS:7 and 9). The present invention is not limited in scope to these genes, however, as one of ordinary skill in the could, using these nucleic acids, readily identify related homologs in various other species (e.g., rat, rabbit, dog, monkey, gibbon, human, chimp, ape, baboon, cow, pig, horse, sheep, cat and other species).

Detail Description Paragraph:
[0094] In addition, it should be clear that the present invention is not limited to the specific nucleic acids disclosed herein. As discussed below, an "MCIP gene" may contain a variety of different bases and yet still produce a corresponding polypeptide that is functionally indistinguishable, and in some cases structurally, from the human and mouse genes disclosed herein.

Detail Description Paragraph:

[0105] Alternatively, the hybridizing segments may be shorter oligonucleotides. Sequences of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 500, 1000, 1212, 1500, 2000, 2500, 3000 or 3431 bases and longer are contemplated as well. Such oligonucleotides will find use, for example, as probes in Southern and Northern blots and as primers in amplification reactions.

Detail Description Paragraph:
[0115] Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject.

Detail Description Paragraph:
[0130] In certain embodiments, the native MCIP promoter will be employed to drive expression of either the corresponding MCIP gene, a heterologous MCIP gene, a screenable or selectable marker gene, or any other gene of interest. Of particular interest is the 700 bp immediately upstream of Exon 4 of the human MCIP gene. As discussed above, this region contains a high concentration of binding sites for the transcription factor NFAT and therefore is likely to play an important regulatory function, especially in light of the existence of a transcript initiating at Exon 4.

Detail Description Paragraph:
[0131] In other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

Detail Description Paragraph:
[0138] Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detail Description Paragraph:
[0147] The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without

potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in humans.

Detail Description Paragraph:
[0150] Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham; et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1 -deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993).

Detail Description Paragraph:
[0151] Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detail Description Paragraph:
[0153] Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detail Description Paragraph:
[0160] A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

Detail Description Paragraph:
[0179] Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide of the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically an animal used for production of anti-antisera is a non-human animal including rabbits, mice, rats, hamsters, pigs or horses. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

Detail Description Paragraph:
[0226] To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the second antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detail Description Paragraph:
[0241] Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks and drugs--in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

Detail Description Paragraph:
[0242] One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Detail Description Paragraph:
[0250] Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Detail Description Paragraph:
[0258] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip)

of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detail Description Paragraph:
[0309] Plasmid Constructs--An HA-tagged splice variant 4 of human MCIP1 (GenBank Accession No. U85267) was isolated from a human placental cDNA library by PCR using the primers:

Detail Description Paragraph:
[0314] GST-MCIP1 fusions were expressed from the bacterial expression plasmid pGEX-CS (T. D. Parks, et al. 1994). Luciferase reporter plasmids, Mb-luc and IL-2-luc, were constructed in pGL3 (Promega) by inserting promoter/enhancer regions from genes encoding human myoglobin (Chin et al., 1998) or IL-2 (Clipstone et al., 1992), respectively. In addition, a synthetic enhancer consisting of three copies of a high affinity MEF2 binding sequence from the desmin promoter (Naya et al., 2000) was linked to a minimal promoter (hsp68) and inserted into pGL3 yielding the des-MEF-luc reporter. The .beta.-galactosidase reporter plasmid pCMV-lacZ (J. Grayson, et al. 1998), and expression vectors encoding constitutively active forms of NFAT (NFAT) (Molkentin et al., 1998), calcineurin (CnA*) (Chin et al., 1998, O'Keefe et al., 1992), or calmodulindependent protein kinase type IV (CaMKIV) (Ho et al., 1996), were previously described. The identity of plasmid constructions was confirmed by restriction mapping and partial DNA sequencing.

Detail Description Paragraph:
[0321] Forced Expression of MCIP1 Blocks Calcineurin-Dependent Transcriptional Activation in Cultured Skeletal Myoblasts and Myotubes--To determine whether MCIP alters calcineurin signaling in mammalian muscle cells, a mouse myoblast cell line, C2C12, was transfected with plasmid DNA constructs expressing human MCIP1 and a constitutively active form of calcineurin (CnA*) (O'Keefe et al., 1992) along with luciferase reporter plasmids controlled either by a minimal promoter (TATA-luc) or two different calcineurin-responsive enhancer constructs from the human interleukin-2 (IL-2-luc) (Clipstone et al., 1992) or myoglobin (Mb-luc) (Chin et al., 1998) genes. The minimal TATA promoter was unresponsive to either CnA* or MCIP1 in both proliferating myoblasts and differentiated myotubes (FIG. 1A). Both the IL-2 and Mb enhancer constructs were activated by CnA*, and this effect was abolished by forced expression of MCIP1. MCIP1 alone had no effect on the activity of any constructs.

Detail Description Paragraph:
[0326] MCIP Inhibits Calcineurin-dependent Regulation of MEF2 --Previous experiments in (Chin et al., 1998), and by others (Liu et al., 1997, Mao et al., 1999, Youn et al., 1999), have indicated that the transactivating function of MEF2 transcription factors, in addition to NFAT proteins, is modified by calcineurin activity. The precise mechanism of this response has not been elucidated, but this interaction can be demonstrated in a myocyte cell background using a MEF2-dependent reporter plasmid (des-MEF2-luc) constructed by linking three copies of a high affinity MEF2 binding site from the human desmin gene to a minimal promoter (Naya et al., 2000). Transcription of the des-MEF2-luc reporter is up-regulated synergistically in skeletal myoblasts by constitutively activated forms of calcineurin (CnA*) and calmodulin-dependent protein kinase IV (CaMKIV*) (FIG. 2C). In a manner similar to its effect on calcineurindependent activation of the myoglobin and IL-2 promoter/reporter constructs (see FIGS. 1A-B), MCIP1 inhibited activation of the des-MEF2-luc reporter by CnA* alone, or by the combination of

CnA* with CaMKIV (FIG. 2C). Since the des-MEF2-luc reporter lacks an NFAT binding site, the inhibitory effect of MCIP observed with this reporter suggests that MCIP acts to inhibit the action of calcineurin on multiple substrates, rather than by interference that is limited to the NFAT:calcineurin interaction.

Detail Description Paragraph:
[0327] Multiple Members of the Family of MCIP Proteins Can Inhibit Calcineurin--The human DSCR1 gene that encodes the protein now termed MCIP1 is composed of seven exons (Fuentes et al., 1997). In humans, there are four splice variants each starting with a different initiating exon (1, 2, 3, or 4), followed by exons 5, 6, and 7. Splice variants 1 and 4 account for the vast majority of detectable transcripts. Exons 1 and 4 each encode the first 29 amino acids of proteins encoded by this gene and are more than 70% identical. Proteins produced from all splicing variations share the regions encoded by exons 5-7. The splice variant of MCIP1 initiated by exon 4 (SEQ ID NOS: 20 and 21) was used in most of the experiments reported here. It has been determined, however, that proteins encoded by human splice variant 1 (SEQ ID NOS:18 and 19), and by murine splice variants 1 and 4 (SEQ ID NOS:12, 13, 14, 15), function similarly to inhibit calcineurin signaling to the myoglobin enhancer in a myocyte cell background (FIG. 3). Likewise, the protein now termed MCIP2, encoded by the ZAKI-4 gene, is 70% identical to MCIP1 and inhibits calcineurin-dependent transcriptional activation in this co-transfection assay (FIG. 3).

Detail Description Paragraph:
[0331] The Subcellular Localization of MCIP1 is Altered by Activated Calcineurin--A GFP-tagged human MCIP1 protein (MCIP-GFP) was expressed in C2C12 myoblasts to assess the subcellular distribution of MCIP1 in this cell background. Twenty hours after transfection, GFP-tagged MCIP1 was localized predominately to the nuclear compartment (FIG. 6A). After 48 h, very little of the MCIP-GFP protein remained. Co-transfection of plasmids encoding CnA* altered this pattern, such that after 24 h MCIP-GFP was observed predominately in the cytoplasm, and a fluorescent signal remained detectable for several days. The morphology of some cells suggested a nearly complete nuclear exclusion of MCIP-GFP in the presence of CnA* (FIG. 6B). Activation of endogenous calcineurin by addition of PMA/ionophore to the medium after transfection also resulted in the accumulation of MCIP-GFP in the cytoplasm (FIG. 6C). The subcellular distribution of native GFP was unaffected by co-expression of calcineurin.

Detail Description Paragraph:
[0333] MCIP1 and MCIP2 are Expressed Most Abundantly in Striated Myocytes, and Their Expression is Up-regulated During Muscle Differentiation--Gene-specific probes complementary to the 3'-untranslated regions of the mouse MCIP1 and MCIP2 cDNAs were used to examine expression of these genes in cultured myogenic cells and in tissues of adult mice. C2C12 myoblasts express low levels of MCIP1 mRNA transcript, but, upon differentiation of these cells into striated myotubes, expression increases severalfold (FIGS. 6A-B). In adult mice, MCIP1 and MCIP2 are expressed most abundantly in heart and skeletal muscles. MCIP2 also is highly expressed in brain, but all other tissues express lower levels of both transcripts (FIGS. 6A-B). These results are consistent with previous descriptions of transcripts derived from the DSCR1 and ZAKI-4 genes in human tissues (Miyazaki et al., 1996, Fuentes et al., 1995).

Detail Description Paragraph:
[0338] Plasmid constructions: The segment of intron 3 from the human MCIP1 (DSCR1) gene was isolated by PCR using human genomic DNA as template and primers based on sequence information from the human Chromosome 21 data bank (29). This .about.900 bp fragment was subcloned into a pGL3 luciferase reporter vector (Promega). Other plasmids were previously described (Chin et al., 1998; Rothermel et al., 2000).

Detail Description Paragraph:

[0347] Thyroid hormone induces expression of MCIP2 but not MCIP1 : The gene encoding MCIP2 was identified originally in a subtractive cloning experiment designed to identify genes that are up-regulated by thyroid hormone in cultured human fibroblasts (Miyazaki et al., 1996). To determine whether MCIP genes are regulated by thyroid hormone in hearts of intact animals, hyperthyroidism was induced in wildtype mice by intraperitoneal injection of T3 for ten days. As noted previously (Robbins & Swain, 1992), T3 treated hearts were uniformly hypertrophic (mean heart weight=180 mg versus 130 mg; mean heart weight/body weight ratio=7.2 mg/gm versus 4.9 mg/gm; n=4 animals in each group). In contrast to the effects of activated calcineurin in the murine heart (FIG. 7), the expression of MCIP 1 was unaltered in hyperthyroid hearts. However, MCIP2 transcript levels were increased approximately 2-fold in both heart and soleus skeletal muscles of T3-treated mice (Table 5, right hand panel). It remains to be determined whether the effects of T3 are a direct consequence of nuclear receptor binding to regulatory elements of the MCIP2 gene, or a result of indirect mechanisms.

Detail Description Paragraph:
[0348] An intragenic region located 5' to exon 4 of the MCIP1 gene is sufficient to promote a transcriptional response to calcineurin: The human MCIP1 gene (annotated initially as DSCR1) was reported to express four variant mRNAs with each of four alternative exons incorporated selectively at the 5' terminus of the expressed transcripts (Fuentes et al., 1997). The majority of these transcripts were identified to represent isoforms that include sequences encoded either by exon 1 or exon 4 (Fuentes et al., 1997). These variants have unique 5' UTR regions, and encode proteins that differ within the first 29 amino acids. The remaining 168 residues of MCIP1, encoded by exons 5-7, are identical in all MCIP1 variants (FIG. 8A). In experiments on hearts of transgenic mice, the inventors determined that expression of the exon 4 variant of MCIP1 mRNA was particularly sensitive to calcineurin activity. The increased abundance of MCIP1 mRNA detected by a probe complementary to the 3' UTR, which is included within all variants of MCIP1 (FIG. 8A), was mirrored by the increase detected with a probe complementary only to unique exon 4 sequences. In contrast, MCIP1 transcripts that include exon 1 sequences were present only at the limit of detection in wildtype murine hearts, and were not induced by the activated calcineurin transgene (not shown).

Detail Description Paragraph:
[0351] Plasmid constructs and generation of transgenic mice: A full length human MCIP1 cDNA encoding the exon 4 splice variant of hMCIP1 with an HA epitope tag from the human influenza hemaglutinin protein (hMCIP1-HA) was cloned 3' to a 5.5 kb segment of the .alpha.-myosin heavy chain promoter (.alpha.-MHC) and 5' to a 0.6 kb polyadenylation signal from the human growth hormone gene (FIG. 10), carried in the pBluescriptII SK+ vector (Stratagene, La Jolla, Calif.). The transgene was linearized and separated from prokaryotic sequences following digestion with NotI, and microinjected into fertilized oocytes from C57/BL6 mice, which were introduced into pseudopregnant females to generate lines of transgenic mice, using standard techniques. Animals were genotyped by Southern blot analysis of tail genomic DNA digested with EcoRI and probed with the hMCIP1 transgene. Animals carrying the .alpha.-MHC-hMCIP1 transgene were crossed with transgenic mice expressing a constitutively activated form of calcineurin, also under the control of the .alpha.-MHC promoter (Gulick et al., 1991) to produce doubly transgenic mice (.alpha.-MHC-hMCIP1.times..alpha.-MHC-CnA*). MCIP expression plasmids were constructed in the pTARGET vector (Promega, Madison, Wis.) under the control of the cytomegalovirus (CMV) promoter. pCMV-hMCIP1 encodes an HA-tagged full length hMCIP1 (amino acids 1-197). pCMV-.DELTA.hMCIP1 encodes an HA-tagged truncated hMCIP1 (amino acids 81 to 197). Other expression vectors and reporter genes have been previously described (Rothermel et al., 2000).

Detail Description Paragraph:
[0354] RNA isolation and analysis: Total RNA was prepared from mouse heart and skeletal muscle using RNAzol (Life Technologies, Rockville, Md.) following the

manufacturer's protocol. Northern blot analysis was performed with 20 mg of total RNA in each lane and probed in Ultrahyb (Ambion, Austin, Tex.) with a DNA fragment from the coding region of the human MCIP 1 transgene. RNA dot blots prepared with 2 mg of total RNA were probed with end-labeled oligonucleotides specific for mouse GAPDH, .alpha.-MHC, .beta.-myosin heavy chain (b-MHC), .alpha.-skeletal actin or atrial natriuretic factor (ANF). Bound probes were detected on a Storm PhosphorImager (Molecular Dynamics, Sunnyvalle, Calif.) and quantified using ImageQuant (version 1.2).

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[0392] Bhavsar P K, Brand N J, Yacoub M H, Barton P J R, "Isolation and characterization of the human cardiac troponin I gene (TNNI3)," Genomics, 35(1):11-23, 1996.

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Detail Description Paragraph:

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Detail Description Paragraph:
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Detail Description Paragraph:
[0451] Gloss, Bernard, Seedorf, and Klock, "The Upstream Regulatory Region of the Human Papilloma Virus-16 Contains an E2 Protein-Independent Enhancer Which is Specific for Cervical Carcinoma Cells and Regulated by Glucocorticoid Hormones," EMBO J., 6:3735, 1987.

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[0455] Goodbourn and Maniatis, "Overlapping Positive and Negative Regulatory Domains of the Human .beta.-Interferon Gene," Proc. Natl. Acad. Sci. USA, 85:1447, 1988.

Detail Description Paragraph:
[0456] Goodbourn, Burstein, and Maniatis, "The Human Beta-Interferon Gene Enhancer is Under Negative Control," Cell, 45:601, 1986.

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☐ 7. Document ID: US 20020123456 A1

L4: Entry 7 of 10 File: PGPB Sep 5, 2002

DOCUMENT-IDENTIFIER: US 20020123456 A1
TITLE: Methods of identifying agents affecting atrophy and hypertrophy

Summary of Invention Paragraph:
[0017] In preferred embodiments of the invention the muscle cells are in a

vertebrate animal having an atrophy-inducing condition, wherein the vertebrate animal is a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate, or human.

Summary of Invention Paragraph:
[0027] An additional embodiment of the invention is a method of inhibiting atrophy in skeletal muscle cells comprising treating the cells with a muscle tissue-specific activator of the PI3K/Akt pathway. In all of the methods herein described the skeletal muscle cells may be in a vertebrate animal having an atrophy-inducing condition. Such vertebrate animal may be a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate, or human and may be treated prior to exposure to or onset of the atrophy-inducing condition. Such atrophy-inducing condition may be immobilization, denervation, starvation, nutritional deficiency, metabolic stress, diabetes, aging, muscular dystrophy, AIDS/HIV infection, cancer, bed rest or myopathy.

Summary of Invention Paragraph:
[0031] The invention also embodies a method of reducing muscle atrophy or inducing muscle hypertrophy in skeletal muscle cells comprising treating the cells with a muscle tissue-specific activator of the PI3K/Akt pathway or inhibitor of the SHIP2 pathway or an inhibitor of SHIP2. Such skeletal muscle cells may be within a vertebrate animal, and such vertebrate animal may be a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate, or human.

Summary of Invention Paragraph:
[0036] An additional embodiment of this invention is a method of treating illnesses, syndromes or disorders associated with muscle atrophy comprising administering to an animal a compound that modulates SHIP2 or the Akt pathway such that symptoms are alleviated. Such animal may be a mammal or a human.

Detail Description Paragraph:
[0052] According to the invention, specific inhibitors of SHIP2 or the SHIP2 pathway are agents that may be used to decrease and/or prevent atrophy in mammals having a condition, such as those described herein, in which skeletal muscle atrophy is occurring. According to this embodiment, atrophying skeletal muscle cells, or vertebrate animals having a condition as described above in which muscle cells are atrophying, are treated with a specific inhibitor of SHIP2 so as to prevent or decrease muscle cell atrophy. Such treatment may be utilized prophylactically prior to the onset of muscle atrophy or after such condition has manifested itself. Vertebrate animals include any species containing skeletal muscle and a backbone, and includes chickens, rodents, rabbits, dogs, cats, cows, horses, pigs, sheep, primates, and humans, preferably humans.

Detail Description Paragraph:
[0055] The activity of the compositions of the invention in vertebrate animals may be assessed using experimental animal models of disorders in which muscle atrophy is present. For example, the activity of the compositions may be tested for their effect in the hindlimb immobilization model described herein in Example 2 infra. Alternatively, the activity of the compositions may be assessed using experimental animals in which hypertrophy can be measured. For example, the activity of the compositions may be tested for their effect on muscles undergoing exercise-induced hypertrophy, or compensation-induced hypertrophy. Alternatively, the muscle may be assessed in control animals as compared to animals treated with the experimental compositions, to determine if the treated animals exhibit skeletal muscle hypertrophy as a result of their treatment. Data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in vertebrate animals, including humans. The dosage of the compositions of the invention should lie within a range of serum circulating concentrations with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration.

Detail Description Paragraph:
[0083] Taken together, the above findings indicate that the Akt/mTOR pathway is activated in, and requisite for, muscle hypertrophy in vivo. To test the hypothesis that activation of mTOR and its downstream targets was not only required for muscle fibre hypertrophy but could actually trigger it, we injected a genetic construct designed to express a constitutively active form of Akt (c.a. Akt) into the tibialis anterior (TA) muscle of adult mice (Eves, E. M. et al. Mol. Cell. Biol. 18, 2143-2152 (1998)). We have demonstrated that the over-expression of c.a. Akt in vitro leads to phosphorylation of p70S6K and PHAS-1/4E-BP1 and to marked hypertrophy of myotubes. The c.a. Akt was fused to enhanced green fluorescent protein (EGFP) and over-expressed under the control of the human skeletal actin promoter (Brennan, K. J. & Hardeman, E. C. J. Biol. Chem. 268, 719-725 (1993)).

Detail Description Paragraph:
[0088] Contrary to previous reports, our data suggest that activation of a calcineurin signaling pathway is not crucial for load-induced hypertrophy of skeletal muscle or the switch to expression of slow MyHC. (Dunn, S. E., Burns, J. L. & Michel, R. N. J. Biol. Chem. 274, 21908-21912 (1999). Dunn, S. E., Chin, E. R. & Michel, R. N. J. Cell Biol. 151, 663-672 (2000).) Our conclusions are based on the findings that cyclosporin was unable to block hypertrophy after 7-30 days of daily administration and that calcineurin activity decreased, as opposed to increased, during the hypertrophy process. These conclusions are in disagreement with those of Dunn et al., who reported that CsA blocked load-induced hypertrophy when delivered at a dose of 25 mg kg-1 twice daily. However, those authors observed a significant decrease in the amount of hypertrophy only at 30 days after the surgical overload, but not at 7 or 14 days while hypertrophy was ongoing and already prominent. Thus, the inability of Dunn et al to block hypertrophy with CsA while it was continuing seems consistent with our conclusion that the calcineurin pathway is not required for the hypertrophy process. Moreover, the late effects of CsA in their hands probably reflect a general toxic effect of long-term, high-dose CsA administration because overall body weight significantly decreased in their long-term-treated animals. The recent findings that tenfold over-expression of activated calcineurin in muscle does not lead to muscle hypertrophy or additional growth after surgical overload, and that treatment with cyclosporin does not prevent IGF-1-mediated hypertrophy further supports the conclusion that calcineurin is not involved in a crucial signaling pathway that is necessary for adaptive hypertrophy of muscle fibres in adult rodents.

Detail Description Paragraph:
[0096] Transfection in vivo. Constructs used encoded the following: (1) myristoylated, c.a. Akt (refs 22, 36) fused in frame at the 3.cndot. end to the gene encoding EGFP (Clontech), and subcloned into an expression vector containing the human skeletal actin (HSA) promoter23; or (2) the gene encoding EGFP alone, subcloned into the same expression vector containing the HSA promoter. The c.a. Akt-EGFP fusion protein was tested in C2C12 myotubes, and mediated the activation of p70s6k and PHAS1/4E-BP1 (data not shown), as expected for c.a. Akt. As an additional control, the myristoylated, HA-tagged c.a. Akt was subcloned into a vector consisting of the CMV promoter. Human inositol 5-phosphatase SHIP2 was cloned from an Origene library. SHIP2 was HA-tagged and subcloned into an expression vector containing the human skeletal actin promoter.

Detail Description Paragraph:
[0107] The idea that calcineurin has a role in skeletal muscle hypertrophy was based largely on findings that either cyclosporin A (CsA; a calcineurin inhibitor) or dominant-negative forms of calcineurin could block IGF-1-induced hypertrophy in muscle cultures (Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N. & Rosenthal, N. Nature 400, 581-585 (1999); Semsarian, C., Sutrave, P., Richmond, D. R. & Graham, R. M. Biochem. J. 339, 443-451 (1999)). However, these findings might have resulted from an inhibition of myoblast differentiation and fusion, as opposed

to direct inhibition of muscle hypertrophy, because the calcineurin blockers were administered to undifferentiated myoblasts, before fusion, and because calcineurin has since been shown to be required for myoblast differentiation. (Musaro, A., McCullagh, K. J., Naya, F. J., Olson, E. N. & Rosenthal, N. Nature 400, 581-585 (1999); Semsarian, C., Sutrave, P., Richmond, D. R. & Graham, R. M. Biochem. J. 339,443-451 (1999); 8. Rommel, C. et al. Science 286, 1738-1741 (1999); Friday, B. B., Horsley, V. & Pavlath, G. K. J. Cell Biol. 149, 657-666 (2000)). The possibility that the PI(3)K/Akt pathway accounts for the hypertrophic actions of IGF-1 has not been explored adequately, although IGF-1 has been shown to activate this pathway (Dudek, H. et al. Science 275, 661-665 (1997)).

Detail Description Paragraph:
[0131] Constitutively active calcineurin (carboxy-terminal deletion mutant encoding amino acid residues 1-398 of calcineurin) tagged with the Flag epitope ([EYKEEEK]2) at the carboxy terminus was generated by the polymerase chain reaction from mouse skeletal muscle complementary DNA (Marathon- Ready; Clontech) and was subsequently subcloned into a tetracycline-inducible internal ribosomal entry site (IRES) bicistronic expression vector (pTRE-Flag-c.a.-calcineurin-IRESEGFP). The reverse tetracycline-controlled transcriptional activator (rtTA) was fused at its C terminus to enhanced blue fluorescence protein (EBFP; Clontech) and subcloned into an expression vector containing the muscle creatine kinase (MCK) promoter8. The tetracycline-responsive vector encoding constitutively active calcineurin and EGFP on the same transcript (as a constitutively active calcineurin-IRESEGFP cassette) was stably transfected into an MCK-rtTAEBFP cell line. Myoblasts harvested after FACS analysis were treated 48 h after the induction of myogenic differentiation with 2 .mu.g ml-1 doxycycline (from a 10 mg ml-1 stock solution in water; SIGMA). At day 4 of differentiation, cell lysates were prepared as described8 and calcineurin was immunoprecipitated with an anti-Flag antibody (Sigma) followed by immunoblot analysis with anti-Flag. Constitutively active Akt was as described previously; it was expressed in a vector containing the MCK promoter and an IRES-EGFP cassette. The kinase-inactive Akt was a gift from the Tsichlis laboratory and was cloned into the same MCK-IRES-EGFP vector. The constitutively active form of p70S6K was a gift from John Blenis's laboratory and was also cloned into the same MCK-IRES-EGFP vector; the kinase activity of the constitutively active p70S6K was determined by transiently transfecting the construct, a wtp70S6K construct and a negative-control vector construct into COS cells, starving those cells and determining the kinase activity as described (data not shown). Human SHIP2 was cloned from an Origene library. The dominant-negative mutant of SHIP2 contained a D690A mutation. SHIP2 was tagged with the haemagglutinin epitope and cloned into the MCK-IRES-EGFP vector.

CLAIMS:

7. The method of claim 6 wherein the vertebrate animal is a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate, or human.

23. The method of claim 22 wherein the vertebrate animal is a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate, or human.

39. The method of claim 37 such that the mammal is a human.

☐ 8. Document ID: US 20020028240 A1

L4: Entry 8 of 10 File: PGPB Mar 7, 2002

DOCUMENT-IDENTIFIER: US 20020028240 A1
TITLE: Timed-release compression-coated solid composition for oral administration

Summary of Invention Paragraph:
[0012] Advantageously, the compositions of the present invention can be used in methods for alleviating undesirable drug interaction between a drug and other drugs used concomitantly that employ the same route for drug absorption, distribution, metabolism or excretion in vivo in humans, especially when the concomitantly used drugs are orally administered.

Detail Description Paragraph:
[0019] The present invention further relates to a method of reducing undesirable pharmacokinetic drug interaction between a drug and another concomitant drug that use the same route for in vivo drug absorption, distribution, metabolism or excretion in humans by using the above-mentioned timed-release compression-coated solid composition for oral administration.

Detail Description Paragraph:
[0026] Specifically, the drug used can be an osteoporosis drug, a bone metabolism-improving agent, a hypnotic sedative, a sleep-inducing agent, an anti-anxiety agent, an anti-epilepsy agent, an antidepressant, an anti-Parkinson's agent, an agent used for the treatment of psychoneurosis, an agent used for the treatment of central nervous system disorders, a local anesthetic, a skeletal muscle relaxant, an agent used in the treatment of autonomic nervous system disorders, an anti-inflammatory antipyretic analgesic, a spasmolytic, an anti-vertigo agent, a cardiotonic, an agent for treatment of arrhythmia, a diuretic, a hypotensive, a vasoconstrictor, a vasodilator, a drug for treatment of circulatory disorders, an agent for hyperlipidemia, an agent that promotes respiration, an antitussive, an expectorant, an antitussive expectorant, a bronchodilator, an antidiarrheal agent, an agent for controlling intestinal function, an agent for treatment of peptic ulcers, an antacid, a laxative, a cholagogue, a gastrointestinal drug, an adrenocortical hormone, a hormone, an agent for treatment of urogenital disorders, a vitamin, a hemostatic, an agent for treating liver disease, an agent for treatment of gout, an agent for treatment of diabetes, an antihistamine, an antibiotic, an antibacterial, an anti-malignant tumor agent, a chemotherapeutic agent, a multisymptom cold agent, a nutrition-enhancing health agent, etc. Examples are bisphosphonate compounds (incadronate, [(cycloheptylamino)-methylene]bis-phosphonate), YM175; produced by the method in Japanese Patent No. Toku Kou Hei 7-629), minodronic acid, [1-hydroxy-2-imidazo(1,2-a)pyridin-3-ylethylidene] bis-phosphonate), YM529; produced by the method entered in Japanese Patent No. Toku Kou Hei 6-99457), alendronate, ibandronate, etidronate, olpadronate, chlodronate, zoledronate, tiludronate, neridronate, pamidronate, risedronate, [1-hydroxy-3-(1-pyrrolidinyl)-propylidene]bis-p- hosphonate, etc.), 5-aminosalicylic acid, acyclovir, adinazolam, ascrobic acid, aspirin, acetylsalicylic acid, acetaminophen, acetobutol, acetohexamide, atenolol, atorvastatin, apomorphine, aminopyrine, aminophylline, ethyl aminobenzoate, amrinone, amobarbital, albuterol, alprazolam, allopurinol, ampicillin, ambroxole isoniazide, idebenone, ibuprofen, indeloxazine, indomethacin, ethenzamide, ethosuccimide, etomidoline, enalapril, ephedrine, erythromycin, oxytetracycline, oxyphenbutazone, osalazine, omeprazole, carmofur, quinidine, glibizide, chloramphenicol, chlordiazepoxide, chlorthiazide, ketoconazole, codeine, cobamamide, colchicine, zafirlukast, diazepam, digitoxin, diclofenac, diclofenac sodium, cyclophosphamide, digoxin, cycotiamine, dipyridamole, cimetidine, josamycine, simvastatin, sucralfate, spironolactone, sulpiride, sulfasalazine, sulfmethoxazole, sulfisoxazole, cefotetan, cefuroxime, selegiline, celecoxib, tasosartan, thiotepa, theophylline, dextromethorphan,

tetracycline, tepronone, terfenadine, terbutaline, doxorubicin, tramadole, etodolac, triamcinolone, triamterene, torbutamide, nadolol, naproxen, nicotinamide, nitroglycerin, nitrofurantoin, nifedipine, nemonapride, noscapine, hydrocortisone, vardecoxib, sodium valproate, haloperidol, hydrochlorothiazide, hydrocortisone, pilocarpine, famotidine, phemacetin, phenytoin, phenylbutazone, phenyl propanolamine, phenobarbital, fenoprofen calcium, pseudoephedrine, budesonide, formoterol fumarate, praunotol, pravastatin, pravastatin sodium, pranrucast, purimidone, fluorouracil, prednisolone, prednisone, procainamide, prostaglandin I derivatives, such as beraprost sodium, etc., furosemide, probenecid, bromvaleryl urea, betamethasone, penicillin, peroxetin, perfphenazine, benzyl penicillin, pentazocine, calcium homopanthothenate, polythiazide, chlorophenylamine maleate, midazolam, milnacidran, doxazocin mesilyate, methyl dopa, methylphenidate, methoclopramide, methotrexate, methoprolol, mepiprizole, morphine, ranitidine, lansoprazole, lisinopril, risperidone, griseofulvin, lidocaine, codeine phosphate, dimemorfan phosphate, pyridoxal phosphate, reserpine, levo dopa, lovastatin, lorazepam, warfarin, aclarubicin hydrochloride, azasetron hydrochloride, amitriptyline hydrochloride, amosulalol hydrochloride, talampicillin hydrochloride, indenolol hydrochloride, ethambutol hydrochloride, ondansetron hydrochloride, granisetron hydrochloride, chloropromazine hydrochloride, diphenhydramine hydrochloride, dibucaine hydrochloride, tamsulasin hydrochloride, thiapride hydrochloride, terazosine hydrochlorice, nicardipine hydrochloride, barnidipine hydrochloride, hydralazine hydrochloride, bifemerane hydrochloride, prazosin hydrochloride, propafenone hydrochloride, moperone hydrochloride, ranitidine hydrochloride, ramosetron hydrochloride, butyl scopolamine bromide, isosorbid nitrate, quinidine nitrate, guanetidine nitrate, thiamine nitrate, tocopherol acetate, chloral hydrate, N -[4-[(1-acetimideyl-4-piperidyl)oxy]phenyl]-N-[(7-amidino-2-naphthyl)meth- yl] sulfamoyl acetate monomethyl sulfonate (produced by the method entered in World Early Disclosure Pamphlet WO96/16940; compound that inhibits active blood coagulation factor X and useful as a blood coagulation-inhibiting agent and preventive and therapeutic agent for blood clots), etc. Other examples are peptides, proteins, and their derivatives that are freely decomposed in the upper digestive tract, such as insulin, calcitonin, angiotensin, vasopressin, desmopressin, LH-RH (leutinizing hormone releasing hormone), somatostatin, glucagon, oxytocin, gastrin, cyclosporin, somatomedin, secretin, h-ANP (human atrial natriuretic peptide), ACTH (adrenocorticotropic hormone), MSH (melanophore-stimulating hormone), .beta.-endorphin, muramyl dipeptide, enkephalin, neurotensin, bombesin, VIP (vasoactive intestinal peptide), CCK-8 (cholecystokinin-8), PTH (parathyroid hormone), CGRP (calcitonin gene-related peptide), TRH (thyrotropin-releasing hormone), endoserin, hGH (human growth hormone), cytokines, such as interluekin, interferon, colony stimulating factor, tumor necrosis factor etc.

Detail Description Paragraph:
[0078] In order for the drug to be releasable in the lower digestive tract of humans, there must be a gelled outer layer at least 2 hours after administration and the outer layer must be further disintegrated or peeled when it reaches the lower digestive tract so that the core tablet is released. Although it varies with the size of the pharmaceutical preparation, the type of polymer substance, the drug and hydrophilic base, their content, etc., the ratio of polymer substance that forms a hydrogel per total pharmaceutical preparation in order to form an outer layer with such properties in a pharmaceutical preparation of 600 mg/tablet or less is approximately 5 to approximately 95 wt % in a preferred embodiment. Approximately 10 to approximately 90 wt % is further preferred. The amount of hydrogel-forming polymer substance added per 1 tablet pharmaceutical preparation is preferably approximately 20 mg/tablet or more, particularly approximately 30 mg/tablet or more. There is a chance that the pharmaceutical preparation will not withstand contractile motion and erosion in the upper digestive tract and that the drug will therefore be released in the upper digestive tract if the amount of hydrogel-forming polymer substance is less than this amount.

CLAIMS:

22. A method for alleviating undesirable drug interaction between a drug and other drugs used concomitantly that employ the same route for drug absorption, distribution, metabolism or excretion in vivo in humans, whereby the composition in claim 1 is orally administered.

23. A method of alleviating undesirable drug interaction with between a drug having the effect of inhibiting drug metabolism in vivo in humans and another drug according to claim 20 used concomitantly, whereby the composition in claim 1 is used.

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TITLE: Methods and compositions relating to HDAC 4 and 5 regulation of cardiac gene expression

Brief Summary Text (12):
As discussed below, there are four lines of evidence that suggest an important role for MEF2 in the control of cardiac hypertrophy. 1) MEF2 regulates many of the fetal cardiac genes that are up-regulated during hypertrophy. 2) MEF2 transcriptional activity is induced by the same signal transduction pathways that control hypertrophy. 3) MEF2C is upregulated in the hearts of human patients with congestive heart failure. 4) MEF2 synergizes with the thyroid hormone receptor to regulate transcription of the .alpha.-MHC gene (Lee et al., 1997) and thyroid hormone is a potent inducer of hypertrophy.

Brief Summary Text (16):
It is clear that the cardiac hypertrophic response is somehow initiated through a Ca.sup.2+ dependent pathway. However, the precise identification of the gene(s) which mediate(s) the hypertrophic response remains elusive. The present invention is directed toward the elucidation of the exact point in the hypertrophic pathway which may be manipulated to achieve beneficial effects on cardiac hypertrophy. In order to develop pharmacologic strategies for treatment of cardiac hypertrophy in humans, it will be important to establish experimental models which accurately reflect the pathological profile of the disease and to identify compositions which regulate or inhibit hypertrophic growth.

Brief Summary Text (23):
The present invention provides further, a method for treating cardiac hypertrophy in an animal comprising providing at least one of HDAC 4 or 5 to cardiac tissue in the animal. The animal may be a human. In other embodiments both HDAC 4 and 5 are provided to cardiac tissue in the animal. In certain embodiments, at least one of HDAC 4 or 5 is provided by transferring an expression cassette encoding HDAC 4 or HDAC 5, under the control of a promoter active in cardiac tissue, into the cardiac tissue. In another embodiment, the expression cassette is a viral expression vector and transferring is achieved by infection of the cardiac tissue with a viral

particle containing the viral expression vector. In particular embodiments, the viral expression vector is derived from adenovirus, retrovirus, adeno-associated virus, herpesvirus or vaccinia virus. In other embodiments of the invention, methods for treating cardiac hypertrophy further comprise the step of administering a traditional coronary heart disease drug formulation to the animal, such as for example, "beta blockers", anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, endothelin antagonists, cytokine inhibitors/blockers, calcium channel blockers, phosphodiesterase inhibitors and angiotensin type 2 antagonists.

Brief Summary Text (28):
In other embodiments, a non-human transgenic animal is provided lacking one or more functional alleles of HDAC 4 or 5. In particular embodiments, the non-human transgenic animal lacks all functional alleles of HDAC 4 and 5. In yet other embodiments, the non-human transgenic animal is selected from the group consisting of mouse, rat, rabbit, sheep, goat and cow and may further comprise a detectable marker gene under the control of MEF2 regulated promoter. In certain embodiments, the MEF2 regulated promoter is a NGFI-B promoter and the detectable marker gene is .beta.-galactosidase, GFP or luciferase.

Brief Summary Text (31):
Also provided is a method for treating cardiac hypertrophy in an animal comprising providing an inhibitor of HDAC phosphorylation to an animal. The is may be a human. The inhibitor may be an inhibitor of Cam kinase, such as KN62. The method may further comprise providing a second pharmaceutical composition to said animal, for example, "beta blockers", anti-hypertensives, cardiotonics, anti-thrombotics, vasodilators, hormone antagonists, endothelin antagonists, cytokine inhibitors/blockers, calcium channel blockers, phosphodiesterase inhibitors and angiotensin type 2 antagonists. The HDAC may be HDAC 4 or HDAC 5.

Detailed Description Text (6):
Thus, in certain embodiments, the present invention provides methods and compositions to identify inhibitors of cardiac hypertrophy, using HDAC 4 and 5 proteins. In particular embodiments, the invention provides methods and compositions to identify modulators of cardiac cell gene expression. In other embodiments, the invention provides methods of identifying a subject at risk of developing cardiac hypertrophy and provides a non-human transgenic animal lacking one or more functional alleles of HDAC 4or5.

Detailed Description Text (14):
There is substantial evidence suggesting that the intracellular Ca.sup.2+ -binding protein, calmodulin, may be a key regulator of cardiac hypertrophy. For example, overexpression of calmodulin in the hearts of transgenic mice induces hypertrophy (Gruver et al., 1993), and treatment of cultured cardiomyocytes with the calmodulin antagonist W-7 prevents hypertrophy in response to .alpha.-adrenergic stimulation and Ca.sup.2+ channel agonists (Sei et al., 1991). Calcineurin and the multifunctional Ca.sup.2+ /calmodulin-dependent protein kinase (CaMK) are well characterized downstream targets of calmodulin regulation. Indeed, activated CaMKII has been shown to induce the hypertrophic-responsive gene atrial natriuretic factor (ANF) in primary cardiomyocytes in vitro and the CaMK inhibitor KN-93 can block the hypertrophic response to endothelin-1 in vitro (Ramirez et al., 1997; Sei et al., 1991; McDonough and Glembotski, 1992). However, the dB isoform of CaMKII, which is the predominant isoform of CaMKII expressed in the heart, does not activate the complete hypertrophic response in vitro and the potential involvement of this signaling pathway in hypertrophic growth vivo has not been investigated. Recently, CaM kinase activity was also reported to be elevated in human failing hearts (Hoch et al., 1999).

Detailed Description Text (22):
Six different HDACs have been cloned from vertebrate organisms. The first three

human HDACs identified were HDAC1, HDAC2 and HDAC3 (termed class I human HDACs). Recently class II human HDACs, HDAC 4, HDAC 5, HDAC6 and HDAC7 (Kao, et al, 2000) have been cloned and identified (Grozinger et al., 1999, incorporated herein by reference). All share homology in a the catalytic region. HDACs 4 and 5 however, have a unique amino-terminal extension not found in other HDACs. This amino-terminal region contains the MEF2-binding domain. The present invention has identified HDACs 4 and 5 as being involved in the regulation of cardiac gene expression and in particular embodiments, repressing MEF2 transcriptional activity. The exact mechanism in which HDAC 4 and HDAC 5 repress MEF2 activity is not completely understood. One possibility is that HDAC 4 or 5 binding to MEF2 inhibits MEF2 transcriptional activity, either competitively or by destabilizing the native, transcriptionally active MEF2 conformation. It is possible also, that HDAC 4 or 5 require dimerization with MEF2 to localize or position HDAC in a proximity to histones for deacetylation to proceed.

Detailed Description Text (86):
In particular embodiments, where clinical application of an active ingredient (drugs, polypeptides, antibodies or liposomes containing oligo- or polynucleotides or expression vectors) is undertaken, it will be necessary to prepare a pharmaceutical composition appropriate for the intended application. Generally, this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

Detailed Description Text (87):
Aqueous compositions of the present invention comprise an effective amount of the active ingredient, as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

Detailed Description Text (90):
The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Detailed Description Text (102):
In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO.sub.2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.5.degree. C. incubator with a humidified atmosphere at 5% CO.sub.2, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

Detailed Description Text (116):
To obtain monoclonal antibodies, one also would immunize an experimental animal, an

antigenic composition. One would then, after a period of time sufficient to allow antibody generation, obtain a population of spleen or lymph cells from the animal. The spleen or lymph cells can then be fused with cell lines, such as human or mouse myeloma strains, to produce antibody-secreting hybridomas. These hybridomas may be isolated to obtain individual clones which can then be screened for production of antibody to the desired target peptide.

Detailed Description Text (126):
Following formation of specific immunocomplexes between the sample and antibody, and subsequent washing, the occurrence and amount of immunocomplex formation may be determined by subjecting the plate to a second antibody probe, the second antibody having specificity for the first (usually the Fc portion of the first is the target). To provide a detecting means, the second antibody will preferably have an associated enzyme that will generate a color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the antibody-bound surface with a urease or peroxidase-conjugated anti-human IgG for a period of time and under conditions which favor the development of immunocomplex formation (e.g., incubation for 2 hr at room temperature in a PBS-containing solution such as PBS/Tween.RTM.).

Detailed Description Text (136):
In certain embodiments, when regulating the expression of genes involved in hypertrophic pathways, it may prove useful to use muscle specific promoters (e.g., human desmin gene promoter, the muscle-specific promoter of the aldolase A gene (pM), smooth muscle .alpha.-actin (SMalphaA) promoter, phosphoglycerate mutase gene (M-PGAM), .alpha.-myosin heavy chain promoter). Cardiac specific promoters include the myosin light chain-2 promoter (Franz et al., 1994; Kelly et al., 1995), the .alpha. actin promoter (Moss et al., 1996), the troponin 1 promoter (Bhavsar et al., 1996); the Na.sup.+ /Ca.sup.2+ exchanger promoter (Barnes et al., 1997), the dystrophin promoter (Kimura et al., 1997), the creatine kinase promoter (Ritchie, M. E., 1996), the alpha7 integrin promoter (Ziober & Kramer, 1996), the brain natriuretic peptide promoter (LaPointe et al., 1996), the alpha B-crystallin/small heat shock protein promoter (Gopal-Srivastava, R., 1995), and alpha myosin heavy chain promoter (Yamauchi-Takihara et al., 1989) and the ANF promoter (LaPointe et al., 1988).

Detailed Description Text (140):
The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

Detailed Description Text (141):
In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter, the Rous sarcoma virus long terminal repeat, .beta.-actin, rat insulin promoter and glyceraldehyde-3-phosphate dehydrogenase can be used to obtain high-level expression of the coding sequence of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a coding sequence of interest is contemplated as well, provided that the levels of expression are sufficient for a given purpose. By employing a promoter with well-known properties, the level and pattern of expression of the protein of interest following transfection or transformation can be optimized.

Detailed Description Text (152):
Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The

nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human or bovine growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Detailed Description Text (170):
The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retroviruses, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. As used herein, the term "genotoxicity" refers to permanent inheritable host cell genetic alteration. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification of normal derivatives. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in non-immunosuppressed humans.

Detailed Description Text (174):
Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1-deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available vectors at high multiplicities of infection (MOI) (Mulligan, 1993; Shenk, 1978).

Detailed Description Text (175):
Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Detailed Description Text (177):
Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical, medical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

Detailed Description Text (184):

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al., 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

Detailed Description Text (189):
HSV, designated with subtypes 1 and 2, are enveloped viruses that are among the most common infectious agents encountered by humans, infecting millions of human subjects worldwide. The large, complex, double-stranded DNA genome encodes for dozens of different gene products, some of which derive from spliced transcripts. In addition to virion and envelope structural components, the virus encodes numerous other proteins including a protease, a ribonucleotides reductase, a DNA polymerase, a ssDNA binding protein, a helicase/primase, a DNA dependent ATPase, a dUTPase and others.

Detailed Description Text (193):
In addition, AAV possesses several unique features that make it more desirable than the other vectors. Unlike retroviruses, AAV can infect non-dividing cells; wild-type AAV has been characterized by integration, in a site-specific manner, into chromosome 19 of human cells (Kotin and Berns, 1989; Kotin et al., 1990; Kotin et al., 1991; Samulski et al., 1991); and AAV also possesses anti-oncogenic properties (Ostrove et al., 1981; Berns and Giraud, 1996). Recombinant AAV genomes are constructed by molecularly cloning DNA sequences of interest between the AAV ITRs, eliminating the entire coding sequences of the wild-type AAV genome. The AAV vectors thus produced lack any of the coding sequences of wild-type AAV, yet retain the property of stable chromosomal integration and expression of the recombinant genes upon transduction both in vitro and in vivo (Berns, 1990; Berns and Bohensky, 1987; Bertran et al., 1996; Kearns et al., 1996; Ponnazhagan et al., 1997a). Until recently, AAV was believed to infect almost all cell types, and even cross species barriers. However, it now has been determined that AAV infection is receptor-mediated (Ponnazhagan et al., 1996; Mizukami et al., 1996).

Detailed Description Text (196):
AAV is not associated with any pathologic state in humans. Interestingly, for efficient replication, AAV requires "helping" functions from viruses such as herpes simplex virus I and II, cytomegalovirus, pseudorabies virus and, of course, adenovirus. The best characterized of the helpers is adenovirus, and many "early" functions for this virus have been shown to assist with AAV replication. Low level expression of AAV rep proteins is believed to hold AAV structural expression in check, and helper virus infection is thought to remove this block.

Detailed Description Text (218):
MEF2 phosphorylation. Phosphopeptide mapping studies demonstrate that MEF2 factors contain multiple phosphorylation sites. It is shown that a casein kinase-I1 (CKII) site in the MADS-box enhances the affinity of MEF2C for DNA (Molkentin et al. 1996c). This site is conserved in all known MEF2 proteins in organisms ranging from Drosophila and C. elegans to humans, consistent with its importance for MEF2 function. It has not yet been determined whether this site is subject to regulated phosphorylation. A schematic diagram of MEF2C and the phosphorylation sites that have been defined to date are shown in FIG. 3.

Detailed Description Text (267):
These experiments have pinpointed the precise molecular details of the mechanism through which hypertrophic signals involving CaMK can activate MEF2. They also suggest an assay for HDAC 5 kinases and for high throughput chemical screens to identify inhibitors of such kinases that are antihypertrophic. A schematic diagram of this type of assay is shown in FIG. 22. According to this assay, the region of

HDAC 5 containing serine residues at 259 and 498 is fused to the GAL4 DNA binding domain and used as bait. This construct can then be expressed in yeast screens in which the GAL4 binding site is used to drive the expression of a LacZ reporter as well as positive or negative selectable markers. Plasmids containing 14-3-3 fused to the GAL4 transcription activation domain would also be introduced into the yeast strain, but they could not associate with HDAC 5 "bait" because serine 259 and 498 within HDAC 5 do not appear to be phosphorylated in yeast. Thus, interaction between the 14-3-3 "prey" and HDAC "bait" would require phosphorylation. Introduction of cDNA libraries from human hearts into yeast will identify kinases that phosphorylate HDAC on the basis of the ability to reconstitute the interaction between 14-3-3 and HDAC. In addition, this same system can be used for high throughput drug screens to identify antihypertrophic compounds that perturb this same interaction.

Detailed Description Text (270):
The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference. U.S. Pat. No. 5,359,046 U.S. Pat. No. 4,367,110 U.S. Pat. No. 4,452,901 U.S. Pat. No. 4,668,621 U.S. Pat. No. 4,873,191 U.S. Pat. No. 5 ,708,158 U.S. Pat. No. 5,252,479 U.S. Pat. No. 5,672,344 WO 84/03564 Adolph et al., "Role of myocyte-specific enhancer-binder factor (MEF-2) in transcriptional regulation of the acardiac myosin heavy chain gene," J. Biol. Chem., 268:5349-5352, 1993. Baichwal and Sugden, In: Gene Transfer, Kucherlapati R, ed., New York, Plenum Press, 117-148, 1986. Batterson and Roizman, J. Virol., 46:371-377, 1983. Bedzyk et al., J. Biol. Chem., 265:18615, 1990. Bellon et al., de Ses Filiales, 190(1):109-142, 1996. Benvenisty and Neshif, Proc. Nat'l Acad. Sci. USA, 83:9551-9555, 1986. Berns and Bohenzky, Adv. Virus Res., 32:243-307, 1987. Berns and Giraud, Curr. Top. Microbiol. Immunol., 218:1-23, 1996. Berns, Microbiol Rev., 54:316-329, 1990. Bertran et al., J. Virol., 70(10):6759-6766, 1996. Bito et al., "CREB Phosphorylation and Dephosphorylation: Aca2+- and Stimulus Duration-Dependent Switch for Hippocampal Gene Expression," Cell,. 87:1203-1214, 1996. Botinelli et a., Circ. Res. 82:106-115, 1997. Bour et al., "Drosophila MEF2, a transcription factor that is essential for myogenesis," Genes and Dev., 9:730-741, 1995. Bowman et al., "Expression of Protein Kinase C B in the Heart Causes Hypertrophy in Adult Mice and Sudden Death in Neonates," J. Clin. Invest., 100:2189-2195, 1997. Brand, "Myocyte enhancer factor 2 (MEF2)," Int J. Biochem. Cell Biol., 29:1467-1470; 1997. Brinster et al., Proc. Nat'l Acad. Sci. USA, 82: 4438-4442, 1985. Brown et al., J. Neurochem. 40:299-308, 1983. Bustamante et al., J. Cardiovasc. Pharmacol, 17: S110-113, 1991. Chaudhary et al., Proc. Nat'l Acad. Sci., 87:9491, 1990. Chen and Okayama, Mol. Cell Biol., 7:2745-2752, 1987. Chien et al., Ann. Rev. Physiol. 55, 77-95, 1993. Chien et al., "Regulation of cardiac gene expression during myocardial growth and hypertrophy: Molecular studies of an adaptive physiologic response," FASEB J., 5:3037-3046, 1991. Chomczynski and Sacchi, Anal. Biochem., 162:156-159, 1987. Clarke et al., "Epidermal Growth Factor Induction of the c-jun Promoter by a Rac Pathway," Mol. Cell Biol., 18:1065-1073, 1998. Coffin, In., Fields BN, Knipe DM, ed. VIROLOGY. New York: Raven Press, pp. 1437-1500, 1990. Colbert et al., "Cardiac Compartment-specific Overexpression of a Modified Retinoic Acid Receptor Produces Dilated Cardiomyopathy and Congestive Heart Failure in Transgenic Mice," J. Clin. Invest., 100: 1958-1968, 1997. Coso et al., "Signaling from G Protein-coupled Receptors to the c-jun promoter Involves the MEF2 Transcription Factor," J. Biol. Chem., 272:20691-20697, 1997. Couch et al., Am. Rev. Resp. Dis., 88:394-403, 1963. DeLuca et al., J. Virol., 56:558-570, 1985. Dolmetsch et al., "Differential activation of transcription factors induced by Ca.sup.2+ response amplitude and duration," Nature, 386:855-858, 1997. Dubensky et al, Proc. Nat'l Acad. Sci. USA, 81:7529-7533, 1984. Edmondson et al., "MEF2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis," Development, 120:1251-1263, 1994. Ellis et al., "Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity," 1986. Elroy-Stein et al., Proc. Nat'l Acad. Sci. USA, 1989. Elshami et al., Gene Therapy, 7(2):141-148, 1996. Emmel et al., "Cyclosporin A specifically inhibits function of nuclear proteins

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**** See image for Certificate of Correction ****
TITLE: Methods for assessing the role of calcineurin immunosuppression and neurotoxicity

Abstract Text (1):
The present invention relates to a method of identifying drugs or agents which have immuno-suppressive effects through or as a result of their effect on calcineurin, including drugs which affect the calcineurin A.alpha. (CNA.alpha.) subunit or the calcineurin A.beta. (CNA.beta.) subunit. In addition, the present invention relates to a method of identifying drugs which reduce (partially or totally) phosphorylation of the microtubule-associated protein tau, in the nervous system of a mammal; a method of identifying drugs which reduce (partially or totally) paired helical filament formation in the nervous system of a mammal; and a method of identifying drugs which reduce (partially or totally) formation of paired helical filaments, amyloid deposits or both. The present invention also relates to

transgenic non-human mammals, such as rodents and particularly mice, which lack a functional calcineurin gene and, thus, have disyrupted calcineurin expression.

Brief Summary Text (2):
Calcineurin, also known as protein phosphatase 2B, was first identified in the bovine brain. It represents a small family of calcium and calmodulin dependent serine/threonine protein phosphatases. It is expressed in all mammalian tissues examined, and is most abundant in the brain. In lymphocytes, calcineurin is the major soluble calmodulin-binding protein. Calcineurin is a heterodimer consisting of a catalytic subunit (A; 61 kD) and a regulatory subunit (B; 19 kD). The A subunit contains a catalytic domain, a carboxyl-terminal inhibitory domain, a B subunit binding site, and a camodulin binding site. The phosphatase activity of the A subunit is regulated by CA.sup.2+ through both calmodulin and the B subunit. The B subunit has only a Ca.sup.2+ dependent regulatory activity and does not have any phosphatase activity. There are two genes encoding closely related (about 80% identical) A subunit isoforms, A.alpha. and A.beta., in the mouse, human, and rat genomes. The .alpha. isoform is the predominant isoform found in brain, thymus, and T cells. The A.alpha. and A.beta. isoforms have distinct cellular distribution in the brain, with A.alpha. most abundant in the hippocampus, cerebral cortex, cerebellum, and striatum. The differential distributions of the two isozymes suggest they may each have specific functions in modulating neuronal activities. The physiologic functions of the different calcineurin A isoforms are not yet defined.

Brief Summary Text (6):
The present invention also relates to transgenic non-human mammals, such as rodents and particularly mice, which lack a functional calcineurin gene and, thus, have disrupted calcineurin expression. In one embodiment, transgenic non-human mammals of the present invention lack a functional calcineurin A.alpha. (CNA.alpha.) subunit gene, a functional calcineurin A.beta. (CNA.beta.) subunit gene or both CNA.alpha. and CNA.beta. subunit genes. In a further embodiment, transgenic non-human mammals (e.g., rodents such as mice and rats) lack a functional calcineurin gene (e.g., calcineurin subunit A.alpha. gene, calcineurin subunit A.beta. gene) and express human tau protein. In such transgenic mammals, hyperphosphorylation of human tau protein is expressed and polymerizes, resulting in formation of paired helical filaments that make up neurofibrillary tangles in the brain. A third type of transgenic non-human mammal (e.g., rodents, such as mice and rats) lacks a functional calcineurin gene, expresses human tau protein and overexpresses human amyloid precursor protein and human Alzheimer A.beta. protein. Such transgenic mammals exhibit both of the pathological lesions of Alzheimer's disease--amyloid deposits and paired helical filaments (which make up the neurofibrillary tangles that accumulate in brain neurons in Alzheimer's disease)--and serve as an improved model for Alzheimer's disease in which to identify drugs or agents which will reduce (partially or totally) the pathological lesions.

Brief Summary Text (8):
As described herein, a transgenic non-human mammal which lacks a functional calcineurin (CN) gene produces greatly increased amounts of hyperphosphorylated tau protein. The transgenic non-human mammal of the present invention can be used to identify drugs or agents which have immuno-suppressive effects through or as a result of their effect on CN, including drugs or agents which affect the calcineurin A.alpha. (CNA.alpha.) subunit or the calcineurin A.beta. (CNA.beta.) subunit. In addition, further transgenic mammals of the present invention, described herein, can be used to identify agents which are useful in reducing phosphorylation of tau protein and production of pathological lesions characteristic of Alzheimer's Disease.

Brief Summary Text (9):
In one embodiment, the present invention relates to a method of identifying an agent that reduces the phosphorylation of tau protein in the nervous system of a

mammal, comprising the steps of a) administering to a transgenic non-human mammal which lacks a functional CN gene, an agent to be assessed for its ability to reduce phosphorylation of tau protein; b) determining the extent to which phosphorylation of tau protein occurs in the nervous system of the transgenic non-human mammal to which the agent is administered; and c) comparing the extent determined in b) to the extent to which phosphorylation occurs in the nervous system of an appropriate control. If phosphorylation occurs to a lesser extent in the nervous system of the transgenic non-human mammal to which the agent is administered than in the nervous system of the control, the agent reduces phosphorylation of tau protein.

Brief Summary Text (10):
In another embodiment, the present invention relates to a method of identifying an agent which reduces paired helical filament (PHF) formation in the nervous system of a mammal, comprising the steps of: a) administering to a transgenic non-human mammal which lacks a functional CN gene and expresses human tau protein, an agent to be assessed for its ability to reduce PHF formation; b) determining the extent to which PHF formation occurs in the nervous system of the transgenic non-human mammal to which the agent is administered; and c) comparing the extent determined in b) to the extent to which PHF formation occurs in the nervous system of an appropriate control, wherein if PHF formation occurs to a lesser extent in the nervous system of the transgenic non-human mammal to which the agent is administered than in the nervous system of the control, the agent reduces PHF formation. In another embodiment, the present invention relates to a method of identifying an agent which reduces a lesion characteristic of Alzheimer's disease in the nervous system of a mammal comprising the steps of: a) administering to a transgenic non-human mammal which lacks a functional CN gene, expresses a human tau protein, and overexpresses the human amyloid precursor protein and the human Alzheimer A.beta. protein, an agent to be assessed for its ability to reduce a lesion characteristic of Alzheimer's disease; b) determining the extent to which the lesion occurs in the nervous system of the transgenic non-human mammal to which the agent is administered; and c) comparing the extent determined in b) to the extent to which the lesion occurs in the nervous system of an appropriate control; wherein if the lesion occurs to a lesser extent in the nervous system of the transgenic non-human mammal to which the agent is administered than in the nervous system of the control, the agent reduces a lesion characteristic of Alzheimer's disease.

Brief Summary Text (12):
The present invention further relates to a method of identifying an agent that reduces the phosphatase activity of calcineurin AP subunit gene in the nervous system of a mammal, comprising the steps of: a) administering to a transgenic non-human mammal which lacks a functional calcineurin A.beta. subunit gene, an agent to be assessed for its ability to reduce the phosphatase activity of calcineurin A.beta. subunit; b) determining the calcineurin A.beta. subunit phosphatase activity present in cells in the nervous system of the transgenic non-human mammal to which the agent is administered; and c) comparing the calcineurin A.beta. phosphatase activity determined in b) to the calcineurin A.beta. phosphatase activity in cells in the nervous system of an appropriate control, wherein if calcineurin A.beta. phosphatase activity is present to a lesser extent in the nervous system of the transgenic non-human mammal to which the agent is administered than in the nervous system of the control, the agent reduces phosphatase activity of calcineurin A.beta. subunit.

Brief Summary Text (13):
The transgenic non-human mammal which lacks a functional CN gene includes mammals in which the CN gene is not present in the genome and mammals in which the structural or functional activity of the CN gene present in the genome of the mammal has been disrupted (both types are referred to as calcineurin knockout mammals). The CNA.alpha. subunit gene and/or the CNA.beta. subunit gene can be removed or functionally disrupted for use in the present invention. For example, as

described in Example 1, the genome of a non-human mammal can be recombined with a sequence which becomes inserted into the exon encoding the CNA.alpha. gene of the animal, resulting in disruption of CNA.alpha. expression. Other methods of producing a CN knockout mammal for use in the present invention can be determined by one of skill in the art using routine experimentation.

Brief Summary Text (14):
A suitable mammal for use in the present invention is a mammal, which upon removal of the CN gene or disruption of the function of the CN gene, produces increased amounts of hyperphosphorylated tau protein. Transgenic non-human mammals of the present invention include rodents, (e.g., rats, mice) and primates.

Brief Summary Text (16):
The methods used to determine the ability of an agent or drug to reduce a lesion characteristic of Alzheimer's disease, which includes phosphorylation of tau protein, are routine methods known to those of skill in the art. For example, as described in Example 1, determination of the extent to which phosphorylation of the tau protein occurs in the transgenic non-human mammal of the present invention can be determined using anti-PHF antibodies. Anti-PHF antibodies can also be used to determine the extent to which PHF formation occurs. Assessing reduction of amyloid deposits can be determined using anti-.beta. protein, thioflavin S or Congo Red. In addition, behavioral observations of the transgenic mammal to which an agent has been administered can be used to determine the ability of the agent to reduce lesions characteristic of Alzheimer's disease, including phosphorylation of tau protein.

Brief Summary Text (17):
A suitable control is a transgenic non-human mammal which has the same characteristics as the transgenic animal to which an agent being assessed is administered (i.e., the test transgenic non-human mammal). The test and control non-human mammals are maintained under the same conditions; they differ only in the presence (test animal) or absence (control animal) of the agent being assessed. For example, a suitable control used to compare the results achieved with the agent or drug to be assessed, is a transgenic non-human mammal which lacks a functional CN gene, a transgenic non-human mammal which lacks a functional CN gene and expresses a human tau protein or a transgenic non-human mammal which lacks a functional CN gene, expresses a human tau protein and overexpresses the human APP and the human Alzheimer A.beta. proteins in the absence of the agent being assessed. For example, in the embodiment for identifying an agent that reduces the phosphorylation of tau protein in the nervous system of a mammal, a suitable control is a transgenic non-human mammal which lacks a functional CN gene. The amount of phosphorylation of tau protein in the control transgenic non-human mammal is determined in the absence of the agent being assessed. Other appropriate controls can be a corresponding wildtype mammal or other control determined by those of skill in the art using no more than routine experimentation.

Brief Summary Text (18):
The present invention further relates to a transgenic non-human mammal which lacks a functional calcineurin gene. In addition, the present invention relates to a transgenic non-human mammal which lacks a functional calcineurin gene and expresses the human tau protein. Further, the present invention relates to a transgenic non-human mammal which lacks a functional calcineurin gene, expresses the human tau protein and overexpresses the human amyloid precursor protein (APP) and the human Alzheimer A.beta. protein.

Brief Summary Text (20):
At the present time, no rodent model exists that generates paired helical filaments or accumulates hyperphosphorylated tau protein. The Exemplar/Athena transgenic mouse model for Alzheimer's disease overexpresses a mutant form of the APP gene (associated with familial Alzheimer's disease) and shows some synaptic loss and the

accumulation of amyloid, but it does not produce paired helical filaments and does not show clear learning disorders (Games, D., et al., Nature, 373:523-527 (1995). It is therefore of importance to develop a mouse model in which paired helical filaments can form. Such a model would serve as a target for testing potential Alzheimer's therapeutic agents designed to reduce or prevent the formation of paired helical filaments. In addition, an animal model, preferably rodent, that shows both amyloid deposits and paired helical filaments would most closely resemble human Alzheimer's disease and would allow the testing of therapeutic agents directed at reducing both of the pathological legions of Alzheimer's disease.

Brief Summary Text (21):
On the basis of the discovery that the calcineurin knockout mouse produces greatly increased amounts of hyperphosphorylated tau protein, the mouse model for Alzheimer's disease in which a hyperphosphorylated form of the human tau protein is expressed, hyperphosphorylated human tau protein and the accumulation of paired helical filaments is exhibited and the Alzheimer amyloid deposits of .beta. protein and the paired helical filaments of hyperphosphorylated tau protein is expressed is generated as described below.

Brief Summary Text (22):
The calcineurin knockout mice is mated to a mouse line homozygous for the expression of a human tau protein. These latter mice have been generated by standard transgenic technology in which the human tau protein is injected into fertilized mouse oocytes in a construct that allows its expression under the control of the human thy 1 promoter. In these animals, the transgenic human tau protein is present in nerve cell bodies, axons, and dendrites and is partially hyperphosphorylated at the appropriate sites for producing paired helical filaments, but not to the degree in the mouse tau protein in the calcineurin knockout mouse line. Mating of these two animals will generate progeny, all of whom will carry a knocked out calcineurin gene on one chromosome, a normal calcineurin gene on the homologous chromosome, and half of whom will carry the human tau transgene.

Brief Summary Text (23):
The genotype of the progeny is determined by removing a small section of the tail, preparing DNA, and carrying out either a Southern Blot or PCR analysis to determine that they all carry one knocked out calcineurin gene and that 50% carry the human tau transgene. The progeny carrying the human tau sequence are grown to adulthood and inter-mated to generate a new set of progeny, 25% of whom are, by Mendelian laws, homozygous for the knocked out calcineurin gene and either homozygous or heterozygous for the human tau transgene. The genotypes of these animals is determined as before by analysis of tail DNA. Animals carrying the knocked out calcineurin gene in homozygous state plus the human tau transgene in either the heterozygous or homozygous state are further analyzed. Animals whose genotypes have been confirmed by the analysis of tail DNA are allowed to reach maturity and inter-mated to generate a line of animals that continues to have the correct genotypes. Mice at different ages are perfused with fixative and subjected to immunocytochemistry and electron microscopy to confirm that they express human tau protein and do not express the calcineurin protein.

Brief Summary Text (24):
Phosphorylation-sensitive antibodies are used as described for the calcineurin knockout mouse to confirm that the human tau protein is hyperphosphorylated due to the lack of calcineurin in its environment. Particular focus is placed on the hippocampus which has previously been shown to be an area of high calcineurin expression and the largest increase in hyperphosphorylation of tau due to the calcineurin knockout mutation. Neurofibrillary tangles are identified by modified Bielchowsky silver stain and by anti-PHF antibodies and are confirmed by electron microscopic identification. The protein expression studies are complemented by

northern blot analysis to confirm that the calcineurin gene in these animals is not expressed.

Brief Summary Text (25):
Once a mouse line has been generated, it expresses hyperphosphorylated human tau protein and preferably paired helical filaments in the neurons of the hippocampus. The mice can be used in several ways. First, they can be used directly to screen for therapeutic agents that reduce the hyperphosphorylation of tau and the production of paired helical filaments. They can also be used to test putative therapeutic agents for their efficacy in preventing the hyperphosphorylation of tau and the formation of paired helical filaments. these mice can also be used to determine the ideal dose of a putative therapeutic agent for Alzheimer's disease.

Brief Summary Text (26):
The mice can also be used to generate a further improved animal model for Alzheimer's disease. For this use, homozygous mice which lack a functional calcineurin gene (mice homozygous for the knocked out calcineurin gene) and which express the human tau gene so that hyperphosphorylated human tau protein, and preferably PHF, is produced in the brain are mated to the Exemplar/Athena APP transgenic mouse that overexpresses the APP protein and the Alzheimer A.beta. protein and, as a consequence, produces amyloid deposits. The purpose of this cross is to generate progeny that have all of the characteristics of Alzheimer's disease, namely hyperphosphorylated tau, paired helical filaments, and amyloid deposits. The progeny of this cross are analyzed as before using tail DNA to confirm their genotype. For example, two heterozygous animals are crossed, one expressing the human tau transgene and one expressing a human APP transgene. Tail DNA analysis is carried out to determine which of the progeny carry both transgenes. If, on the other hand, the mating is between a homozygous version of the APP transgenic mouse and a homozygous version of the human tau transgenic mouse (of course already combined with the homozygous calcineurin knocked mutation), then technically the tail DNA analysis should not be necessary but is carried out nonetheless in case the germ line of any of the mice has lot any of the transgenes. The progeny of this cross thus carry two human transgenes, one for APP and one for tau under different promoters but both expressed in the nervous system, plus a homozygous knockout mutation in the calcineurin gene.

Detailed Description Text (24):
The amount of calcineurin A.alpha. in T cell extracts was assessed by Western blotting using standard procedures. Twenty micrograms of T cell extract or brain homogenates were fractionated by SDS-PAGE on a 16% Tris/glycine gel (Novex) at 150 volts (constant voltage)and transferred to a PVDF membrane (Immobilon) at 100 volts for 1.5 hours. Following transfer, the membrane was blocked in M-Blotto overnight at 4.degree. C. The membranes were briefly rinsed in PBS and reacted with either rabbit antibody R2929 (specific for C-terminal peptide, SNSSNIQ (SEQ ID NO.:1) from human CNA.alpha.) or rabbit antibody R2948 (specific for CNA.beta. residues 386-396, LMTEGEDEFDG (SEQ ID NO.:2)). Rabbit anti-peptide antiserum was diluted to 1:10,000. The membranes were washed and incubated for 1 hour at room temperature in TBST containing HRP-conjugated donkey-anti-rabbit secondary antibody (Amersham) diluted 1:10,000. The membranes were washed in TBST and developed with the ECL Western blotting detection system (Amersham).

Detailed Description Text (149):
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Detailed Description Text (177):

Tau gained special attention when it was shown that the neurofibrillary tangles that accumulate in Alzheimer's disease are composed predominantly of tau that is abnormally phosphorylated. Using antibodies against hyperphosphorylated tau, changes were detected much earlier than the development of argyrophilic staining and before the appearance of any NFT or amyloid plaques (Braak et al., 1994; Braak et al., 1994). Indeed, phosphorylation of tau has also been shown to alter its structure by making it longer and stiffer (Hagestedt et al., 1989). These findings prompted research into possible mechanisms by which tau could become hyperphosphorylated. As mentioned above, several kinases will phosphorylate tau to generate PHF-like immunoreactive isoforms and several phosphatases, including calcineurin will return PHF tau to a more normal state of phosphorylation. Calcineurin has been further implicated recently, with other phosphatases, in the formation of PHF tau by studies on biopsied human brain tissue, in which it was demonstrated that tau is normally phosphorylated but becomes dephosphorylated during tissue processing and preparation by calcineurin and other phosphatases present in normal but not Alzheimer's brains (Matsuo et al., 1994). These findings were taken to indicate that in Alzheimer's disease deficits in phosphatases such as calcineurin might underlie the accumulation of hyperphosphorylated tau. Another study that examined the levels of calcineurin in Alzheimer's and normal cerebellum and neocortex immunocytochemically found no differences in protein level between the two groups. However, calcineurin was located around some neurofibrillary tangles, and the study did not compare levels of calcineurin enzyme activity (Billingsley et al., 1994).

Detailed Description Text (183):

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Detailed Description Paragraph Table (2):
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Other Reference Publication (10):
Pearson, B.E. and Choi, T.K., "Expression of the human .beta.-amyloid precursor protein gene from a yeast artificial chromosome in transgenic mice," Proc. Natl. Acad. Sci., USA, 90:10578-10582 (1993).

Other Reference Publication (14):
Kawabata, S., et al., "Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein", Nature, 354:476-478 (1991).

Other Reference Publication (28):
Sandhu, F.A. et al., "Expression of the human B-amyloid protein of Alzheimer's disease specifically in the brains of transgenic mice", J. Biol. Chem., 266 (32):21331-21334 (1991).

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Gotz, J., et al., "Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform", EMBO J., 14(7):1304-1313 (1995).

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Cooke, Michael P.	Del Mar	CA	US	

US-CL-CURRENT: 435/456; 435/372

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 2. Document ID: US 20040210950 A1

L7: Entry 2 of 29 File: PGPB Oct 21, 2004

PGPUB-DOCUMENT-NUMBER: 20040210950
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040210950 A1

TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (CALSARCINS)

PUBLICATION-DATE: October 21, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Olson, Eric	Dallas	TX	US	
Frey, Norbert	Dallas	TX	US	

US-CL-CURRENT: 800/8; 435/320.1, 435/325, 435/69.1, 530/350, 536/23.5

ABSTRACT:

The present invention relates to the polypeptides known as calcineurin associated protein (calsarcin). Calsarcins-1, -2, and -3 bind to calcineurin, telethonin, and a-actinin, which provides a link between these molecules and the sarcomere. Sarcomeric dysfunction ultimately leads to activation of calcineurin and consequent hypertrophic cardiomyopathy. Thus, methods utilizing calsarcin as it regards these medical conditions are herein provided and include screening for peptides which interact with calsarcin, screening for modulators of calsarcin binding to calcineurin or .alpha.-actinin, methods to modulate calcineurin activity, methods to inhibit calcineurin activation of gene transcription and methods for treating cardiac hypertrophy, heart failure and Type II diabetes.

☐ 3. Document ID: US 20040186275 A1

L7: Entry 3 of 29 File: PGPB Sep 23, 2004

PGPUB-DOCUMENT-NUMBER: 20040186275
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040186275 A1

TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

PUBLICATION-DATE: September 23, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Olson, Eric	Dallas	TX	US	
Frey, Norbert	Dallas	TX	US	

US-CL-CURRENT: 530/350; 435/320.1, 435/325, 435/69.1, 536/23.5

ABSTRACT:

The present invention relates to the polypeptides known as calcineurin associated protein (calsarcin). Calsarcins-1, -2, and -3 bind to calcineurin, telethonin, and .alpha.-actinin, which provides a link between these molecules and the sarcomere. Sarcomeric dysfunction ultimately leads to activation of calcineurin and consequent hypertrophic cardiomyopathy. Thus, methods utilizing calsarcin as it regards these medical conditions are herein provided and include screening for peptides which interact with calsarcin, screening for modulators of calsarcin binding to calcineurin or .alpha.-actinin, methods to modulate calcineurin activity, methods to inhibit calcineurin activation of gene transcription and methods for treating cardiac hypertrophy, heart failure and Type II diabetes.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 4. Document ID: US 20040127686 A1

L7: Entry 4 of 29 File: PGPB Jul 1, 2004

PGPUB-DOCUMENT-NUMBER: 20040127686
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040127686 A1

TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

PUBLICATION-DATE: July 1, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Olson, Eric	Dallas	TX	US	
Frey, Norbert	Dallas	TX	US	

US-CL-CURRENT: 530/350; 435/320.1, 435/325, 435/69.1, 536/23.5

ABSTRACT:

The present invention relates to the polypeptides known as calcineurin associated protein (calsarcin). Calsarcins-1, -2, and -3 bind to calcineurin, telethonin, and .alpha.-actinin, which provides a link between these molecules and the sarcomere. Sarcomeric dysfunction ultimately leads to activation of calcineurin and consequent hypertrophic cardiomyopathy. Thus, methods utilizing calsarcin as it regards these medical conditions are herein provided and include screening for peptides which interact with calsarcin, screening for modulators of calsarcin binding to calcineurin or .alpha.-actinin, methods to modulate calcineurin activity, methods to inhibit calcineurin activation of gene transcription and methods for treating cardiac hypertrophy, heart failure and Type II diabetes.

☐ 5. Document ID: US 20040087564 A1

L7: Entry 5 of 29 File: PGPB May 6, 2004

PGPUB-DOCUMENT-NUMBER: 20040087564
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20040087564 A1

TITLE: Delivery composition and method

PUBLICATION-DATE: May 6, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wright, D. Craig	Pacific Grove	CA	US	
Mauk, John E.	Arlington	VA	US	

US-CL-CURRENT: 514/179

ABSTRACT:

A composition which includes a membrane modulators is disclosed. The composition can be used in a wide range of therapies for delivering a membrane modulator which play an active function in regulating, controlling or causing a desired therapeutic effect to a target cell.

☐ 6. Document ID: US 20030186913 A1

L7: Entry 6 of 29 File: PGPB Oct 2, 2003

PGPUB-DOCUMENT-NUMBER: 20030186913
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030186913 A1

TITLE: Expression of exogenous polynucleotide sequences in a vertebrate

PUBLICATION-DATE: October 2, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Wolff, Jon A.	Madison	WI	US	
Duke, David J.	Salem	OR	US	
Felgner, Philip L.	Rancho Santa Fe	CA	US	

US-CL-CURRENT: 514/44

ABSTRACT:

The present invention provides a method for delivering a pharmaceutical polypeptide to the interior of a cardiac cell of a vertebrate in vivo, comprising the step of introducing a preparation comprising a pharmaceutically acceptable injectable carrier and naked polynucleotide operatively coding for the polypeptide into the interstitial space of the heart, whereby the naked polynucleotide is taken up into the interior of the cell and has a pharmacological effect on the vertebrate. In a preferred embodiment wherein the polynucleotide encodes polypeptide immunologically foreign to the vertebrate, the delivery method preferably comprises delivering an immunosuppressive agent to the vertebrate to limit immune responses directed to the polypeptide.

☐ 7. Document ID: US 20030113301 A1

L7: Entry 7 of 29 File: PGPB Jun 19, 2003

PGPUB-DOCUMENT-NUMBER: 20030113301
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030113301 A1

TITLE: Muscle cells and their use in cardiac repair

PUBLICATION-DATE: June 19, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Edge, Albert	Cambridge	MA	US	
Dinsmore, Jonathan	Brookline	MA	US	

US-CL-CURRENT: 424/93.21; 424/93.7

ABSTRACT:

Muscle cells and methods for using the muscle cells are provided. In one embodiment, the invention provides transplantable skeletal muscle cell compositions and their methods of use. In one embodiment, the muscle cells can be transplanted into patients having disorders characterized by insufficient cardiac function, e.g., congestive heart failure, in a subject by administering the skeletal myoblasts to the subject. The muscle cells can be autologous, allogeneic, or xenogeneic to the recipient.

☐ 8. Document ID: US 20030078376 A1

L7: Entry 8 of 29 File: PGPB Apr 24, 2003

PGPUB-DOCUMENT-NUMBER: 20030078376
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20030078376 A1

TITLE: Methods and compositions relating to muscle specific sarcomeric calcineurin-binding proteins (calsarcins)

PUBLICATION-DATE: April 24, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Olson, Eric	Dallas	TX	US	
Frey, Norbert	Dallas	TX	US	

US-CL-CURRENT: 530/350

ABSTRACT:

The present invention relates to the polypeptides known as calcineurin associated protein (calsarcin). Calsarcins-1, -2, and -3 bind to calcineurin, telethonin, and .alpha.-actinin, which provides a link between these molecules and the sarcomere. Sarcomeric dysfunction ultimately leads to activation of calcineurin and consequent hypertrophic cardiomyopathy. Thus, methods utilizing calsarcin as it regards these medical conditions are herein provided and include screening for peptides which interact with calsarcin, screening for modulators of calsarcin

binding to calcineurin or .alpha.-actinin, methods to modulate calcineurin activity, methods to inhibit calcineurin activation of gene transcription and methods for treating cardiac hypertrophy, heart failure and Type II diabetes.

☐ 9. Document ID: US 20020164702 A1

L7: Entry 9 of 29 File: PGPB Nov 7, 2002

PGPUB-DOCUMENT-NUMBER: 20020164702
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020164702 A1

TITLE: Novel tyrosine kinase receptors and ligands

PUBLICATION-DATE: November 7, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Valenzuela, David M.	Yorktown Heights	NY	US	
Glass, David J.	Cortlandt Manor	NY	US	
Bowen, David C.	Washington	DC	US	
Yancopoulos, George D.	Yorktown Heights	NY	US	

US-CL-CURRENT: 435/69.1; 435/183, 435/320.1, 435/325, 530/350, 536/23.2

ABSTRACT:

The present invention provides for a gene, designated as musk, that encodes a novel tyrosine kinase receptor expressed in high levels in denervated muscle. The invention also provides for an isolated polypeptide which activates MuSK receptor. The invention further provides for a polypeptide which is functionally equivalent to the MuSK activating polypeptide. The invention also provides assay systems that may be used to detect and/or measure ligands that bind the musk gene product. The present invention also provides for diagnostic and therapeutic methods based on molecules that activate MuSK.

☐ 10. Document ID: US 20020150953 A1

L7: Entry 10 of 29 File: PGPB Oct 17, 2002

PGPUB-DOCUMENT-NUMBER: 20020150953
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020150953 A1

TITLE: Methods and compositions relating to muscle selective calcineurin interacting protein (MCIP)

PUBLICATION-DATE: October 17, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Williams, R. Sanders	Dallas	TX	US	
Rothermel, Beverly	Bedford	TX	US	

US-CL-CURRENT: 435/7.23; 424/9.2, 435/7.92

ABSTRACT:

The present invention relates to the polypeptides known as muscle calcineurin interacting proteins (MCIPs). These molecules binding to calcineurin and, in so doing, modulate its functions, which includes phosphate removal as part of a pathway coupling Ca.sup.2+ to cellular responses in muscle. MCIPs form a physical complex with the catalytic subunit of calcineurin, and increased levels of MCIPs correspond to a reduced ability of calcineurin to stimulate transcription of certain target genes. Methods to exploit these observation are provided and include screening for modulators of MCIP expression and binding to calcineurin, methods of diagnosis of MCIP defects, and methods for treating cardiomyopathies, including cardiac hypertropy.

☐ 11. Document ID: US 20020123456 A1

L7: Entry 11 of 29 File: PGPB Sep 5, 2002

PGPUB-DOCUMENT-NUMBER: 20020123456
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020123456 A1

TITLE: Methods of identifying agents affecting atrophy and hypertrophy

PUBLICATION-DATE: September 5, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Glass, David J.	Cortlandt Manor	NY	US	

US-CL-CURRENT: 514/1; 435/7.2

ABSTRACT:

The present invention provides a method for inhibiting atrophy or causing hypertrophy in muscle cells, by inhibiting the action of the phosphatase SHIP2. It further provides a method for inhibiting skeletal muscle atrophy or causing skeletal muscle hypertrophy in vertebrate animals, by inhibiting the Akt pathway through the inhibition of SHIP2. It also provides a method of identifying agents that may be used for inhibiting atrophy or causing hypertrophy in muscle cells, by screening for inhibitors of SHIP2 or inhibitors of the SHIP2 signaling pathway.

☐ 12. Document ID: US 20020028240 A1

L7: Entry 12 of 29 File: PGPB Mar 7, 2002

PGPUB-DOCUMENT-NUMBER: 20020028240
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20020028240 A1

TITLE: Timed-release compression-coated solid composition for oral administration

PUBLICATION-DATE: March 7, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
Sawada, Toyohiro	Fujieda-shi		JP	
Sako, Kazuhiro	Yaizu-shi		JP	
Yoshioka, Tatsunobu	Yaizu-shi		JP	
Watanabe, Shunsuke	Fujieda-shi		JP	

US-CL-CURRENT: 424/472; 514/215

ABSTRACT:

The present invention was completed based on these discoveries and relates to in a hydrogel-forming compression-coated solid pharmaceutical preparation comprising a core tablet containing drug and outer layer made from hydrogel-forming polymer substance and hydrophilic base, the improvement, a timed-release compression-coated solid composition for oral administration, said composition comprising (1) drug and freely erodible filler are mixed with the core tablet, (2) the percentage erosion of the core tablet is approximately 40 to approximately 90%, and (3) the outer layer essentially does not contain the same drug as the above-mentioned drug. By releasing a drug after a specific lag time, it becomes possible to effectively deliver a drug to a specific site in the digestive tract. It is therefore useful as presented as a timed-release solid composition for oral administration of a drug that is to be effectively delivered in high concentrations to the afflicted site in the lower digestive tract, a drug that is to be effectively absorbed in the lower digestive tract, a drug that is effective for chronopharmacotherapy, etc.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

☐ 13. Document ID: US 6852838 B2

L7: Entry 13 of 29 File: USPT Feb 8, 2005

US-PAT-NO: 6852838
DOCUMENT-IDENTIFIER: US 6852838 B2

TITLE: Tyrosine kinase receptors and ligands

DATE-ISSUED: February 8, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Valenzuela; David M.	Yorktown Heights	NY		
Glass; David J.	Cortlandt Manor	NY		
Bowen; David C.	Washington	DC		
Yancopoulos; George D.	Yorktown Heights	NY		

US-CL-CURRENT: 530/350

ABSTRACT:

The present invention provides for a gene, designated as musk, that encodes a novel tyrosine kinase receptor expressed in high levels in denervated muscle. The invention also provides for an isolated polypeptide which activates MuSK receptor. The invention further provides for a polypeptide which is functionally equivalent to the MuSK activating polypeptide. The invention also provides assay systems that may be used to detect and/or measure ligands that bind the musk gene product. The present invention also provides for diagnostic and therapeutic methods based on molecules that activate MuSK.

3 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 24

☐ 14. Document ID: US 6706694 B1

L7: Entry 14 of 29 File: USPT Mar 16, 2004

US-PAT-NO: 6706694
DOCUMENT-IDENTIFIER: US 6706694 B1
**** See image for Certificate of Correction ****

TITLE: Expression of exogenous polynucleotide sequences in a vertebrate

DATE-ISSUED: March 16, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Wolff; Jon A.	Madison	WI		
Duke; David J.	Salem	OR		
Felgner; Philip L.	Rancho Santa Fe	CA		

US-CL-CURRENT: 514/44; 424/130.1, 424/184.1, 435/320.1, 435/325, 435/455

ABSTRACT:

The present invention provides a method for delivering a pharmaceutical polypeptide to the interior of a cardiac cell of a vertebrate in vivo, comprising the step of

introducing a preparation comprising a pharmaceutically acceptable injectable carrier and naked polynucleotide operatively coding for the polypeptide into the interstitial space of the heart, whereby the naked polynucleotide is taken up into the interior of the cell and has a pharmacological effect on the vertebrate. In a preferred embodiment wherein the polynucleotide encodes polypeptide immunologically foreign to the vertebrate, the delivery method preferably comprises delivering an immunosuppressive agent to the vertebrate to limit immune responses directed to the polypeptide.

17 Claims, 15 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 9

☐ 15. Document ID: US 6673604 B1

L7: Entry 15 of 29 File: USPT Jan 6, 2004

US-PAT-NO: 6673604
DOCUMENT-IDENTIFIER: US 6673604 B1

TITLE: Muscle cells and their use in cardiac repair

DATE-ISSUED: January 6, 2004

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Edge; Albert	Cambridge	MA		

US-CL-CURRENT: 435/347; 435/325, 435/371

ABSTRACT:

Muscle cells and methods for using the muscle cells are provided. In one embodiment, the invention provides transplantable skeletal muscle cell compositions and their methods of use. In one embodiment, the muscle cells can be transplanted into patients having disorders characterized by insufficient cardiac function, e.g., congestive heart failure, in a subject by administering the skeletal myoblasts to the subject. The muscle cells can be autologous, allogeneic, or xenogeneic to the recipient.

5 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

☐ 16. Document ID: US 6632628 B1

L7: Entry 16 of 29 File: USPT Oct 14, 2003

US-PAT-NO: 6632628
DOCUMENT-IDENTIFIER: US 6632628 B1
**** See image for Certificate of Correction ****

TITLE: Methods and compositions relating to HDAC 4 and 5 regulation of cardiac gene expression

DATE-ISSUED: October 14, 2003

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Olson; Eric N.	Dallas	TX		
Lu; Jianrong	Quincy	MA		
McKinsey; Timothy	Dallas	TX		

US-CL-CURRENT: 435/18; 435/29, 514/44

ABSTRACT:

The present invention relates to cardiac hypertrophy. More particularly, the present invention defines the molecular events linking calcium stimulation to cardiac hypertrophy. More specifically, the present invention shows that Ca.sup.2+ stimulation of the hypertrophic response is mediated through an HDAC 4 and 5 interaction with MEF2, and that phosphorylation of HDACs results in loss of HDAC-mediated repression of MEF2 hypertrophic action. Thus, the present invention provides methods and compositions of treating cardiac hypertrophy, as well as methods and compositions for identifying subjects at risk for cardiac hypertrophy. Further provided are methods for the detection of compounds having therapeutic activity toward cardiac hypertrophy.

21 Claims, 26 Drawing figures
Exemplary Claim Number: 9
Number of Drawing Sheets: 22

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWMC | Draw De

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☐ 17. Document ID: US 6444870 B1

L7: Entry 17 of 29 File: USPT Sep 3, 2002

US-PAT-NO: 6444870
DOCUMENT-IDENTIFIER: US 6444870 B1
**** See image for Certificate of Correction ****

TITLE: Methods for assessing the role of calcineurin immunosuppression and neurotoxicity

DATE-ISSUED: September 3, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Zhang; Wei	Stanford	CA		

Seidman; Jonathan G.	Milton	MA
Kagyali; Usamah S.	Somerville	MA
Potter; Huntington	Boston	MA

US-CL-CURRENT: 800/3; 435/320.1, 435/325, 435/455, 435/463, 800/18, 800/25

ABSTRACT:

The present invention relates to a method of identifying drugs or agents which have immuno-suppressive effects through or as a result of their effect on calcineurin, including drugs which affect the calcineurin A.alpha. (CNA.alpha.) subunit or the calcineurin A.beta. (CNA.beta.) subunit. In addition, the present invention relates to a method of identifying drugs which reduce (partially or totally) phosphorylation of the microtubule-associated protein tau, in the nervous system of a mammal; a method of identifying drugs which reduce (partially or totally) paired helical filament formation in the nervous system of a mammal; and a method of identifying drugs which reduce (partially or totally) formation of paired helical filaments, amyloid deposits or both. The present invention also relates to transgenic non-human mammals, such as rodents and particularly mice, which lack a functional calcineurin gene and, thus, have disyrupted calcineurin expression.

5 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 18. Document ID: US 6413740 B1

L7: Entry 18 of 29 File: USPT Jul 2, 2002

US-PAT-NO: 6413740
DOCUMENT-IDENTIFIER: US 6413740 B1

TITLE: Tyrosine kinase receptors and ligands

DATE-ISSUED: July 2, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Valenzuela; David M.	Yorktown Heights	NY		
Glass; David J.	Cortlandt Manor	NY		
Bowen; David C.	Washington	DC		
Yancopoulos; George D.	Yorktown Heights	NY		

US-CL-CURRENT: 435/69.1; 435/70.1, 435/71.1, 435/71.2, 530/350, 536/23.5

ABSTRACT:

The present invention provides for a gene, designated as musk, that encodes a novel tyrosine kinase receptor expressed in high levels in denervated muscle. The invention also provides for an isolated polypeptide which activates MuSK receptor. The invention further provides for a polypeptide which is functionally equivalent

to the MuSK activating polypeptide. The invention also provides assay systems that may be used to detect and/or measure ligands that bind the musk gene product. The present invention also provides for diagnostic and therapeutic methods based on molecules that activate MuSK.

14 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 24

☐ 19. Document ID: US 6261601 B1

L7: Entry 19 of 29 File: USPT Jul 17, 2001

US-PAT-NO: 6261601
DOCUMENT-IDENTIFIER: US 6261601 B1

TITLE: Orally administered controlled drug delivery system providing temporal and spatial control

DATE-ISSUED: July 17, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Talwar; Naresh	New Delhi			IN
Sen; Himadri	Haryana			IN
Staniforth; John N.	Bath			GB

US-CL-CURRENT: 424/469; 424/43, 424/44, 424/452, 424/465, 424/466, 424/468, 424/484, 424/485, 424/486, 424/488

ABSTRACT:

A pharmaceutical composition in the form of tablets or capsules provides a combination of temporal and spatial control of drug delivery to a patient for effective therapeutic results. The pharmaceutical composition comprises a drug, a gas generating component, a swelling agent, a viscolyzing agent, and optionally a gel forming polymer. The swelling agent belongs to a class of compounds known as superdisintegrants (e.g., cross-linked polyvinylpyrrolidone or sodium carboxymethylcellulose). The viscolyzing agent initially and the gel forming polymer thereafter form a hydrated gel matrix which entraps the gas, causing the tablet or capsule to be retained in the stomach or upper part of the small intestine (spatial control). At the same time, the hydrated gel matrix creates a tortuous diffusion path for the drug, resulting in sustained release of the drug (temporal control). A preferred once daily ciprofloxacin formulation comprises 69.9% ciprofloxacin base, 0.34% sodium alginate, 1.03% xanthan gum, 13.7% sodium bicarbonate, 12.1% cross-linked polyvinylpyrrolidone, and optionally other pharmaceutical excipients, the formulation being in the form of a coated or uncoated tablet or capsule.

11 Claims, 3 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 3

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 20. Document ID: US 6228844 B1

L7: Entry 20 of 29 File: USPT May 8, 2001

US-PAT-NO: 6228844
DOCUMENT-IDENTIFIER: US 6228844 B1

TITLE: Stimulating vascular growth by administration of DNA sequences encoding VEGF

DATE-ISSUED: May 8, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Wolff; Jon A.	Madison	WI		
Duke; David J.	Salem	OR		
Felgner; Philip L.	Rancho Santa Fe	CA		

US-CL-CURRENT: 514/44; 435/455

ABSTRACT:

The present invention provides a method for delivering a pharmaceutical polypeptide to the interior of a cardiac cell of a vertebrate in vivo, comprising the step of introducing a preparation comprising a pharmaceutically acceptable injectable carrier and naked polynucleotide operatively coding for the polypeptide into the interstitial space of the heart, whereby the naked polynucleotide is taken up into the interior of the cell and has a pharmacological effect on the vertebrate such as inducing vascular growth. In a preferred embodiment wherein the polynucleotide encodes polypeptide immunologically foreign to the vertebrate, the delivery method preferably comprises delivering an immunosuppressive agent to the vertebrate to limit immune responses directed to the polypeptide.

27 Claims, 15 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 9

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 21. Document ID: US 6211234 B1

L7: Entry 21 of 29 File: USPT Apr 3, 2001

US-PAT-NO: 6211234
DOCUMENT-IDENTIFIER: US 6211234 B1

TITLE: Substituted phenyl compounds

DATE-ISSUED: April 3, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Astles; Peter Charles	Dagenham			GB
Harper; Mark Francis	Dagenham			GB
Harris; Neil Victor	Dagenham			GB
McLay; Iain McFarlane	Dagenham			GB
Walsh; Roger John Aitchison	Dagenham			GB
Lewis; Richard Alan	Dagenham			GB
Smith; Christopher	Dagenham			GB
Porter; Barry	Dagenham			GB
McCarthy; Clive	Dagenham			GB

US-CL-CURRENT: 514/520; 514/521, 514/522, 514/568, 514/570, 558/411, 558/414, 558/415, 558/423

ABSTRACT:

##STR1##

Compounds of formula (I) are described wherein R.sup.1 is hydrogen, -(lower alkyl).sub.q (CO.sub.2 R.sup.6 or OH), --CN, --C(R.sup.7).dbd.NOR.sup.8, NO.sub.2, --O(lower alkyl)R.sup.9, --C.tbd.C--R.sup.10, --CR.sup.11.dbd.C(R.sup.12) (R.sup.13), --C(.dbd.O)CH.sub.2 C(.dbd.O)CO.sub.2 H, --CO(R.sup.14), alkylthio, alkylsulphinyl, alkylsulphonyl, carbamoyl, thiocarbamoyl, substituted carbamoyl, substituted thiocarbamoyl, sulphamoyl or an optionally substituted nitrogen-containing ring, m, n, o and p are independently zero or 1 and R.sup.2, R.sup.3, R.sup.4 and R.sup.5 are various groups; and physiologically acceptable salts, N-oxides and prodrugs thereof. The compounds have endothelin antagonist activity and are useful as pharmaceuticals.

20 Claims, 0 Drawing figures
Exemplary Claim Number: 1

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 22. Document ID: US 6124343 A

L7: Entry 22 of 29 File: USPT Sep 26, 2000

US-PAT-NO: 6124343
DOCUMENT-IDENTIFIER: US 6124343 A

TITLE: Substituted phenyl compounds with a substituent having a thienyl ring

DATE-ISSUED: September 26, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Smith; Christopher	Dagenham			GB

Porter; Barry	Dagenham	GB
Walsh; Roger	Dagenham	GB
Majid; Tahir	Dagenham	GB
McCarthy; Clive	Dagenham	GB
Harris; Neil	Dagenham	GB
Astles; Peter	Dagenham	GB
McLay; Iain	Dagenham	GB
Morley; Andrew	Dagenham	GB
Bridge; Andrew	Dagenham	GB
Van Sickle; Andrew	Dagenham	GB
Halley; Frank	Dagenham	GB
Roach; Alan	Dagenham	GB
Foster; Martyn	Dagenham	GB

US-CL-CURRENT: 514/438; 549/77, 549/78, 549/79

ABSTRACT:

This invention is directed to compounds of formula I ##STR1## wherein R.sup.1 is CN, CH.sub.2 CN, CH.dbd.CHCN, CHO, or CH.dbd.CHCO.sub.2 H;

R.sup.2 is aryl lower alkoxy, heteroaryl lower alkoxy, aryl lower alkylthio or heteroaryl lower alkylthio wherein each of the aryl and heteroaryl moieties is optionally substituted;

R.sup.3 is halogen;

R.sup.4 is optionally substituted aryl or optionally substituted heteroaryl;

R.sup.5 is carboxy or an acid isostere;

X is oxygen or sulphur; and

n is zero or 1; or an N-oxide thereof, prodrug thereof solvate thereof, or pharmaceutically acceptable salt thereof, which compounds have endothelin antagonist activity. The invention is also directed to methods for preparing the compounds of formula I and their pharmaceutical use.

19 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 23. Document ID: US 6048893 A

L7: Entry 23 of 29 File: USPT Apr 11, 2000

US-PAT-NO: 6048893
DOCUMENT-IDENTIFIER: US 6048893 A

TITLE: Substituted phenyl compounds with a substituent having A 1,3-benzodioxole ring

DATE-ISSUED: April 11, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Smith; Christopher	Dagenham			GB
Porter; Barry	Dagenham			GB
Walsh; Roger	Dagenham			GB
Majid; Tahir	Dagenham			GB
McCarthy; Clive	Dagenham			GB
Harris; Neil	Dagenham			GB
Astles; Peter	Dagenham			GB
McLay; Iain	Dagenham			GB
Morley; Andrew	Dagenham			GB
Bridge; Andrew	Dagenham			GB
Van Sickle; Andrew	Dagenham			GB
Halley; Frank	Dagenham			GB
Roach; Alan	Dagenham			GB
Foster; Martyn	Dagenham			GB

US-CL-CURRENT: 514/466; 549/442, 549/445

ABSTRACT:

This invention is directed to compounds of formula I ##STR1## wherein R.sup.1 is CN, CH.sub.2 CN, CH.dbd.CHCN, CHO, or CH.dbd.CHCO.sub.2 H;

R.sup.2 is aryl lower alkoxy, heteroaryl lower alkoxy, aryl lower alkylthio or heteroaryl lower alkylthio wherein each of the aryl and heteroaryl moieties is optionally substituted;

R.sup.3 is halogen;

R.sup.4 is optionally substituted aryl or optionally substituted heteroaryl;

R.sup.5 is carboxy or an acid isostere;

X is oxygen or sulphur; and

n is zero or 1; or an N-oxide thereof, prodrug thereof solvate thereof, or pharmaceutically acceptable salt thereof, which compounds have endothelin antagonist activity. The invention is also directed to methods for preparing the compounds of formula I and their pharmaceutical use.

23 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 24. Document ID: US 5693622 A

L7: Entry 24 of 29 File: USPT Dec 2, 1997

US-PAT-NO: 5693622
DOCUMENT-IDENTIFIER: US 5693622 A
**** See image for Certificate of Correction ****

TITLE: Expression of exogenous polynucleotide sequences cardiac muscle of a mammal

DATE-ISSUED: December 2, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Wolff; Jon A.	Madison	WI		
Duke; David J.	Salem	OR		
Felgner; Philip L.	Rancho Santa Fe	CA		

US-CL-CURRENT: 514/44

ABSTRACT:

The present invention provides a method for delivering a pharmaceutical polypeptide to the interior of a cardiac cell of a vertebrate in vivo, comprising the step of introducing a preparation comprising a pharmaceutically acceptable injectable carrier and naked polynucleotide operatively coding for the polypeptide into the interstitial space of the heart, whereby the naked polynucleotide is taken up into the interior of the cell and has a pharmacological effect on the vertebrate. In a preferred embodiment wherein the polynucleotide encodes polypeptide immunologically foreign to the vertebrate, the delivery method preferably comprises delivering an immunosuppressive agent to the vertebrate to limit immune responses directed to the polypeptide.

23 Claims, 16 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 9

☐ 25. Document ID: US 5455042 A

L7: Entry 25 of 29 File: USPT Oct 3, 1995

US-PAT-NO: 5455042
DOCUMENT-IDENTIFIER: US 5455042 A

TITLE: Ointment comprising a homogenous mixture of a polymer or copolymer of N-vinylacetamide, water and/or alcohols, and a pharmacologically active component

DATE-ISSUED: October 3, 1995

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sakai; Yasuyuki	Tokyo			JP
Suzuki; Noriyuki	Oita			JP
Kudo; Tetsuo	Oita			JP

Marumo; Kuniomi	Oita	JP
Aizawa; Toshiyuki	Oita	JP
Imamura; Kunio	Tokyo	JP
Sugita; Shuichi	Tokyo	JP
Kanbayashi; Kazuo	Tokyo	JP

US-CL-CURRENT: 424/443; 424/445, 424/447, 424/78.31, 424/78.35, 424/78.37

ABSTRACT:

The subject invention relates to an ointment comprising a homogeneous mixture of polymer or copolymer of N-vinylacetamide, as an essential component, water and/or alcohols, and a pharmacologically active component. The ointment may be coated or plastered onto the skin or mucosa of a human being or animal in order to effect a therapeutic response.

4 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 26. Document ID: US 5344655 A

L7: Entry 26 of 29 File: USPT Sep 6, 1994

US-PAT-NO: 5344655
DOCUMENT-IDENTIFIER: US 5344655 A

TITLE: External application base or auxiliary agent and external application composition for human being or animal containing the same

DATE-ISSUED: September 6, 1994

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sakai; Yasuyuki	Tokyo			JP
Suzuki; Noriyuki	Oita			JP
Kudo; Tetsuo	Oita			JP
Marumo; Kuniomi	Oita			JP
Aizawa; Toshiyuki	Oita			JP
Imamura; Kunio	Tokyo			JP
Sugita; Shuichi	Tokyo			JP
Kanbayashi; Kazuo	Tokyo			JP

US-CL-CURRENT: 424/443; 424/447, 424/78.31, 424/78.35, 424/78.37

ABSTRACT:

A (co)polymer suitable for bases or auxiliary agents for external application (for example, hydrophilic gels, adhesives, thickeners or excipients) to be coated or plastered onto the skin or mucosa of a human being or animal, such as ointment

agents (ointment, hydrogel, jelly or cream), plastering agents (molded poultice, tape agent or plaster agent), sticky bandages (sticky bandage, strap, wound strap, surgical tape, taping material, supporter), and to preparations for external application containing same.

3 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 27. Document ID: US 5254338 A

L7: Entry 27 of 29 File: USPT Oct 19, 1993

US-PAT-NO: 5254338
DOCUMENT-IDENTIFIER: US 5254338 A

TITLE: External application base or auxiliary agent and external application composition for human being or animal containing the same

DATE-ISSUED: October 19, 1993

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Sakai; Yasuyuki	Tokyo			JP
Suzuki; Noriyuki	Oita			JP
Kudo; Tetsuo	Oita			JP
Marumo; Kuniomi	Oita			JP
Aizawa; Toshiyuki	Oita			JP
Imamura; Kunio	Tokyo			JP
Sugita; Shuichi	Tokyo			JP
Kanbayashi; Kazuo	Tokyo			JP

US-CL-CURRENT: 424/78.35; 424/443, 424/447, 424/78.31, 424/78.37

ABSTRACT:

A (co)polymer suitable for bases or auxiliary agents for external application (for example, hydrophilic gels, adhesives, thickeners or excipients) to be coated or plastered onto the skin or mucosa of a human being or animal, such as ointment agents (ointment, hydrogel, jelly or cream), plastering agents (molded poultice, tape agent or plaster agent), sticky bandages (sticky bandage, strap, wound strap, surgical tape, taping material, supporter), and to preparations for external application containing same.

2 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 28. Document ID: US 5139481 A

L7: Entry 28 of 29 File: USPT Aug 18, 1992

US-PAT-NO: 5139481
DOCUMENT-IDENTIFIER: US 5139481 A
**** See image for Certificate of Correction ****

TITLE: Treatment for type II diabetes

DATE-ISSUED: August 18, 1992

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Faustman; Denise	Weston	MA		
Avruch; Joseph	Brookline	MA		

US-CL-CURRENT: 604/522

ABSTRACT:

Disclosed is a method for treating insulin resistance in a patient which involves isolating a skeletal muscle cell from an insulin-sensitive donor and transplanting the skeletal muscle cell into the skeletal muscle tissue of the insulin-resistant patient. The transplanted cell may be a muscle precursor cell, e.g., a myoblast or it may be derived from whole skeletal muscle tissue.

12 Claims, 0 Drawing figures
Exemplary Claim Number: 1

☐ 29. Document ID: US 5130141 A

L7: Entry 29 of 29 File: USPT Jul 14, 1992

US-PAT-NO: 5130141
DOCUMENT-IDENTIFIER: US 5130141 A

TITLE: Compositions for and methods of treating muscle degeneration and weakness

DATE-ISSUED: July 14, 1992

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Law; Peter K.	Memphis	TN	38104	
Goodwin; Tena G.	Memphis	TN	38127	

US-CL-CURRENT: 424/548; 514/11, 514/907

ABSTRACT:

Compositions for and methods of treating muscle weakness and degeneration are described. Such compositions include myogenic cells which are administered by the described methods to one or more affected muscles.

22 Claims, 13 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 2

Term	Documents
HUMAN	512642
HUMANS	144340
(5 AND HUMAN).PGPB,USPT,USOC.	29
(L5 AND HUMAN).PGPB,USPT,USOC.	29

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